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(54) Title: MANIPULATION OF PROTOPORPHYRINOGEN OXIDASE ENZYME ACTIVITY IN EUKARYOTIC ORGANISMS

(57) Abstract

The present invention provides novel eukaryotic DNA sequences coding for native protoporphyrinogen oxidase (protox) or modified forms of the enzyme which are herbicide tolerant. Plants having altered protox activity which confers tolerance to herbicides are also provided. These plants may be bred or engineered for resistance to protox inhibitors *via* mutation of the native protox gene to a resistant form or through increased levels of expression of the native protox gene, or they may be transformed with modified eukaryotic or prokaryotic protox coding sequences or wild type prokaryotic protox sequences which are herbicide tolerant. Diagnostic and other uses for the novel eukaryotic protox sequence are also described. Plant genes encoding wild-type and altered protox, purified plant protox, methods of isolating protox from plants, and methods of using protox-encoding genes are also disclosed.

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MANIPULATION OF PROTOPORPHYRINOGEN OXIDASE ENZYME ACTIVITY IN EUKARYOTIC ORGANISMS

The invention relates generally to the manipulation of the enzymatic activity responsible for the conversion of protoporphyrinogen IX to protoporphyrin IX in a biosynthetic pathway common to all eukaryotic organisms. In one aspect, the invention is applied to the development of herbicide resistance in plants, plant tissues and seeds. In another aspect, the invention is applied to the development of diagnostics and treatments for deficiencies in this enzymatic activity in animals, particularly humans.

The biosynthetic pathways which leads to the production of chlorophyll and heme share a number of common steps. Chlorophyll is a light harvesting pigment present in all green photosynthetic organisms. Heme is a cofactor of hemoglobin, cytochromes, P450 mixed-function oxygenases, peroxidases, and catalases (*see, e.g.* Lehninger, Biochemistry. Worth Publishers, New York (1975)), and is therefore a necessary component for all aerobic organisms.

The last common step in chlorophyll and heme biosynthesis is the oxidation of protoporphyrinogen IX to protoporphyrin IX. Protoporphyrinogen oxidase (referred to herein as "protox") is the enzyme which catalyzes this last oxidation step (Matringe *et al., Biochem. J. 260:* 231 (1989)).

The protox enzyme has been purified either partially or completely from a number of organisms including the yeast *Saccharomyces cerevisiae* (Labbe-Bois and Labbe, In Biosynthesis of Heme and Chlorophyll, E.H. Dailey, ed. McGraw Hill: New York, pp. 235-285 (1990)), barley etioplasts (Jacobs and Jacobs, *Biochem. J. 244:* 219 (1987)), and mouse liver (Dailey and Karr, *Biochem. 26*: 2697 (1987)). Genes encoding protox have been isolated from two prokaryotic organisms, *Escherichia coli* (Sasarman *et al., Can. J. Microbiol. 39:* 1155 (1993)) and *Bacillus subtilis* (Dailey *et al., J. Biol. Chem. 269:* 813 (1994)). These genes share no sequence similarity; neither do their predicted protein products share any amino acid sequence identity. The *E. coli* protein is

approximately 21 kDa, and associates with the cell membrane. The *B. subtilis* protein is 51 kDa, and is a soluble, cytoplasmic activity.

Presently, too little is known about the protox enzyme to allow isolation of protox encoding genes from higher eukaryotic organisms (i.e. animals, plants and all other multicellular nucleate organisms other than lower eukaryotic microorganisms such as yeast, unicellular algae, protozoans, etc.) using known approaches.

In particular, many of the standard techniques for isolation of new proteins and genes are based upon the assumption that they will be significantly similar in primary structure (i.e. amino acid and DNA sequence) to known proteins and genes that have the same function. Such standard techniques include nucleic acid hybridization and amplification by polymerase chain reaction using oligonucleotide primers corresponding to conserved amino acid sequence motifs. These techniques would not be expected to be useful for isolation of eukaryotic protox genes using present structural information which is limited to prokaryotic protox genes since there is no significant structural similarity even among the known prokaryotic protox genes and proteins.

Another approach that has been used to isolate biosynthetic genes in other metabolic pathways from higher eukaryotes is the complementation of microbial mutants deficient in the activity of interest. For this approach, a library of cDNAs from the higher eukaryote is cloned in a vector that can direct expression of the cDNA in the microbial host. The vector is then transformed or otherwise introduced into the mutant microbe, and colonies are selected that are phenotypically no longer mutant.

This strategy has worked for isolating genes from higher eukaryotes that are involved in several metabolic pathways, including histidine biosynthesis (*e.g.* U.S. patent no 5290926 and WO 94/026909 to Ward *et al.*, incorporated by reference herein in its entirety), lysine biosynthesis (e.g. Frisch *et al., Mol. Gen. Genet. 228*: 287 (1991)), purine biosynthesis (e.g. Aimi *et al., J. Biol. Chem. 265*: 9011 (1990)), and tryptophan biosynthesis (e.g. Niyogi *et al., Plant Cell 5:* 1011 (1993)). However, despite the availability of microbial mutants thought to be defective in protox activity (e.g. *E. coli* (Sasarman *et al., J. Gen. Microbiol. 113:* 297 (1979)), *Salmonella typhimurium* (Xu *et al., J. Bacteriol. 174:* 3953 (1992)), and *Saccharomyces cerevisiae* (Camadro *et al,. Biochem. Biophys. Res. Comm. 106*: 724 (1982)), application of this technique to

isolate cDNAs encoding eukaryotic protox enzymatic activity is at best unpredictable based on the available information.

There are several reasons for this. First, the eukaryotic protox cDNA sequence may not be expressed at adequate levels in the mutant microbe, for instance because of codon usage inconsistent with the usage preferences of the microbial host. Second, the primary translation product from the cloned eukaryotic coding sequence may not produce a functional polypeptide, for instance if activity requires a post-translational modification, such as glycosylation, that is not carried out by the microbe. Third, the eukaryotic protein may fail to assume its active conformation in the microbial host, for instance if the protein is normally targeted to a specific organellar membrane system that the microbial host specifically lacks. This last possibility is especially likely for the plant protox enzyme, which is associated in the plant cell with organelles not present in microbial hosts used in the complementation assay. In particular, the plant protox enzyme is associated with both the chloroplast envelope and thylakoid membranes (Matringe *et al., J. Biol. Chem. 267*:4646 (1992)), and presumably reaches those membrane systems as a result of a post-translational targeting mechanism involving both an N-terminal transit sequence, and intrinsic properties of the mature polypeptide (see, e.g. Kohorn and Tobin, *Plant Cell 1:* 159 (1989); Li *et al., Plant Cell 3:* 709 (1991); Li *et al., J. Biol. Chem. 267:* 18999 (1992)).

The protox enzyme is known to play a role in certain human disease conditions. Patients suffering from variegate porphyria, an autosomal dominant disorder characterized by both neuropsychiatric symptoms and skin lesions, have decreased levels of protox activity (Brenner and Bloomer, *New Engl. J. Med. 302:* 765 (1980)). Due to the lack of knowledge regarding the human protox enzyme and its corresponding gene, options for diagnosing and treating this disorder are presently very limited.

The use of herbicides to control undesirable vegetation such as weeds or plants in crops has become almost a universal practice. The relevant market exceeds a billion

dollars annually. Despite this extensive use, weed control remains a significant and costly problem for farmers.

Effective use of herbicides requires sound management. For instance, time and method of application and stage of weed plant development are critical to getting good weed control with herbicides. Since various weed species are resistant to herbicides, the production of effective herbicides becomes increasingly important.

Unfortunately, herbicides that exhibit greater potency, broader weed spectrum and more rapid degradation in soil can also have greater crop phytotoxicity. One solution applied to this problem has been to develop crops which are resistant or tolerant to herbicides. Crop hybrids or varieties resistant to the herbicides allow for the use of the herbicides without attendant risk of damage to the crop. Development of resistance can allow application of a herbicide to a crop where its use was previously precluded or limited (*e.g.* to pre-emergence use) due to sensitivity of the crop to the herbicide. For example, U.S. Patent No. 4,761,373 to Anderson *et al.* is directed to plants resistant to various imidazolinone or sulfonamide herbicides. The resistance is conferred by an altered acetohydroxyacid synthase (AHAS) enzyme. U.S.Patent No. 4,975,374 to Goodman *et al.* relates to plant cells and plants containing a gene encoding a mutant glutamine synthetase (GS) resistant to inhibition by herbicides that were known to inhibit GS, *e.g.* phosphinothricin and methionine sulfoximine. U.S. Patent No. 5,013,659 to Bedbrook *et al.* is directed to plants that express a mutant acetolactate synthase which renders the plants resistant to inhibition by sulfonylurea herbicides. U.S. Patent No. 5,162,602 to Somers *et al.* discloses plants tolerant to inhibition by cyclohexanedione and aryloxyphenoxypropanoic acid herbicides. The tolerance is conferred by an altered acetyl coenzyme A carboxylase(ACCase).

The protox enzyme serves as the target for a variety of herbicidal compounds. The herbicides that inhibit protox include many different structural classes of molecules (Duke *et al., Weed Sci. 39*: 465 (1991); Nandihalli *et al., Pesticide Biochem. Physiol. 43:* 193 (1992); Matringe *et al., FEBS Lett. 245:* 35 (1989); Yanase and Andoh, *Pesticide Biochem. Physiol. 35*: 70 (1989)). These herbicidal compounds include the diphenylethers {e.g. acifluorfen, 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobezoic acid; its methyl ester; or oxyfluorfen, 2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-

(trifluorobenzene)}, oxidiazoles, (e.g. oxidiazon, 3-[2,4-dichloro-5-(1-methylethoxy)phenyl]-5-(1,1-dimethylethyl)-1,3,4-oxadiazol-2-(3*H*)-one), cyclic imides (e.g. S-23142, *N*-(4-chloro-2-fluoro-5-propargyloxyphenyl)-3,4,5,6-tetrahydrophthalimide; chlorophthalim, *N*-(4-chlorophenyl)-3,4,5,6-tetrahydrophthalimide), phenyl pyrazoles (e.g. TNPP-ethyl, ethyl 2-[1-(2,3,4-trichlorophenyl)-4-nitropyrazolyl-5-oxy]propionate; M&B 39279), pyridine derivatives (e.g. LS 82-556), and phenopylate and its *O*-phenylpyrrolidino- and piperidinocarbamate analogs. Many of these compounds competitively inhibit the normal reaction catalyzed by the enzyme, apparently acting as substrate analogs.

The predicted mode of action of protox-inhibiting herbicides involves the accumulation of protoporphyrinogen IX in the chloroplast. This accumulation is thought to lead to leakage of protoporphyrinogen IX into the cytosol where it is oxidized by a peroxidase activity to protoporphyrin IX. When exposed to light, protoporphyrin IX can cause formation of singlet oxygen in the cytosol. This singlet oxygen can in turn lead to the formation of other reactive oxygen species, which can cause lipid peroxidation and membrane disruption leading to rapid cell death (Lee *et al., Plant Physiol. 102*: 881 (1993)).

Not all protox enzymes are sensitive to herbicides which inhibit plant protox enzymes. Both of the protox enzymes encoded by genes isolated from *Escherichia coli* (Sasarman *et al., Can. J. Microbiol. 39:* 1155 (1993)) and *Bacillus subtilis* (Dailey *et al., J. Biol. Chem. 269*: 813 (1994)) are resistant to these herbicidal inhibitors. In addition, mutants of the unicellular alga *Chlamydomonas reinhardtii* resistant to the phenylimide herbicide S-23142 have been reported (Kataoka *et al., J. Pesticide Sci. 15:* 449 (1990); Shibata *et al.*, In Research in Photosynthesis, Vol.III, N. Murata, ed. Kluwer:Netherlands. pp. 567-570 (1992)). At least one of these mutants appears to have an altered protox activity that is resistant not only to the herbicidal inhibitor on which the mutant was selected, but also to other classes of protox inhibitors (Oshio *et al., Z. Naturforsch. 48c:* 339 (1993); Sato *et al.*, In ACS Symposium on Porphyric Pesticides, S. Duke, ed. ACS Press: Washington, D.C. (1994)). A mutant tobacco cell line has also been reported that is resistant to the inhibitor S-21432 (Che *et al.,. Z. Naturforsch. 48c:* 350 (1993).

The present invention provides an isolated DNA molecule encoding the protoporphyrinogen oxidase (protox) enzyme from a eukaryotic organism, which preferably is a higher eukaryotic organism. In particular, the present invention provides isolated DNA molecules encoding the protoporphyrinogen oxidase (protox) enzyme from a plant or human source.

Preferred within the scope of the invention are isolated DNA molecules encoding the protoporphyrinogen oxidase (protox) enzyme from dicotyledonous plants, but especially from *Arabidopsis* plants, such as those given in SEQ ID NOS: 1, 3, and 9.

Also preferred are isolated DNA molecules encoding the protoporphyrinogen oxidase (protox) enzyme from monocotyledonous plants, but especially from maize plants, such as those given in SEQ ID NOS: 5 and 7. Especially preferred within the invention is an isolated DNA molecule encoding the protoporphyrinogen oxidase (protox) enzyme protein from a dicotyledonous plant, wherein said protein comprises the amino acid sequence selected from the group consisting of SEQ ID Nos. 2, 4 and 10. Also preferred is a an isolated DNA molecule encoding the protoporphyrinogen oxidase (protox) enzyme protein from a monocotyledonous plant, wherein said protein comprises the amino acid sequence selected from the group consisting of SEQ ID Nos. 6 and 8.

Using the information provided by the present invention, the DNA coding sequence for the protoporphyrinogen oxidase (protox) enzyme from any eukaryotic organism may be obtained using standard methods. Thus, in a further embodiment the present invention provides probes capable of specifically hybridizing to a eukaryotic DNA ~~sequence encoding a protoporphyrinogen oxidase activity or to the respective mRNA~~ and methods for detecting the said DNA sequences in eucaryotic organisms using the probes according to the invention.

The present invention further enbodies expression cassetts and recombinant vectors comprising the said expression cassetts comprising essentially a promoter, but especially a promoter that is active in a plant, operably linked to a DNA molecule encoding the protoporphyrinogen oxidase (protox) enzyme from a eukaryotic organism

according to the invention. The expression cassette according to the invention may in addition further comprise a signal sequence operably linked to said DNA molecule, wherein said signal sequence is capable of targeting the protein encoded by said DNA molecule into the chloroplast or the mitochondria.

In addition, the present invention provides plants, plant cells, plant tissues and plant seeds with altered protox activity which are resistant or at least tolerant to inhibition by a herbicide at levels which normally are inhibitory to the naturally occurring protox activity in the plant. In particular, the invention embodies plants, wherein the altered protox activity is conferred by over-expression of the wild-type protox enzyme or by expression of a DNA molecule encoding a herbicide tolerant protox enzyme. The said herbicide tolerant protox enzyme may be a modified form of a protox enzyme that naturally occurs in a eukaryote or a prokaryote; or a modified form of a protox enzyme that naturally occurs in said plant; or the said herbicide tolerant protox enzyme may naturally occur in a prokaryote. Plants encompassed by the invention include monocotyledonous and dicotyledonous plants, but especially hybrid plants, Preferred are those plants which would be potential targets for protox inhibiting herbicides, particularly agronomically important crops such as maize and other cereal crops such as wheat, oats, rye, sorghum, rice, barley, millet, turf and forage grasses, and the like, as well as cotton, tobacco, sugar cane, sugar beet, oilseed rape, and soybeans.

The present invention further encompasses propagating material of a plant according to the invention, preferably plant seed, treated with a protectant coating, but especially a protectant coating comprising a preparation selected from the group consisting of herbicides, insecticides, fungicides, bactericides, nematicides, molluscicides or mixtures thereof.

The present invention is further directed to methods for the production of plants, plant cells, plant tissues, and plant seeds and the transgenic progeny thereof which contain a protox enzyme resistant to, or tolerant of inhibition by a herbicide at a concentration which inhibits the naturally occurring protox activity. The said resistance or tolerance may be obtained by expressing in the said transgenic plants either a DNA molecule encoding a modified form of a protox enzyme that naturally occurs in a karyote, or a modified form of a protox enzyme that naturally occurs in said plant, or

a protox enzyme that naturally occurs in a prokaryot, or a protox enzyme which is a modified form of a protein which naturally occurs in a prokaryote.

One specific embodiment of the invention is directed to the preparation of transgenic maize plants, maize tissue or maize seed and the transgenic progeny thereof which have been stably transformed with a recombinant DNA molecule comprising a suitable promoter functional in plants operably linked to a structural gene encoding an unmodified prokaryotic protox enzyme which is resistant to the herbicide.

The invention is further directed to the preparation of transgenic plants, plant cells, plant tissue and plant seed and the transgenic progeny thereof which has been stably transformed with a recombinant DNA molecule comprising a suitable promoter functional in plants operably linked to a structural gene encoding an unmodified eukaryotic protox enzyme. This results in over-expression of the unmodified protox in the plant sufficient to overcome inhibition of the enzyme by the herbicide.

The present invention also embodies the production of plants which express an altered protox enzyme tolerant of inhibition by a herbicide at a concentration which normally inhibits the activity of wild-type, unaltered protox. In this embodiment, the plant may be stably transformed with a recombinant DNA molecule comprising a structural gene encoding the resistant protox, or prepared by direct selection techniques whereby herbicide resistant lines are isolated, characterized and developed.

The present invention is further directed to a method for controlling the growth of undesired vegetation which comprises applying to a population of a plant with altered protox activity which is resistant to inhibition by a herbicide at levels which normally are inhibitory to the naturally occurring protox activity in the said plant, an effective amount of a protox-inhibiting herbicide. Plants to be protected in the described way are especially those which would be potential targets for protox inhibiting herbicides, particularly agronomically important crops such as, for example, maize and other cereal crops such as wheat, oats, rye, sorghum, rice, barley, millet, turf and forage grasses, and the like, as well as cotton, sugar cane, sugar beet, oilseed rape, and soybeans

Herbicides that qualify as protox inhibitors are those selected from the group consisting of aryluracil, diphenylether, oxidiazole, imide, phenyl pyrazole, pyridine derivative, phenopylate and *O*-phenylpyrrolidino- and piperidinocarbamate analogs of said phenopylate.

The present invention also embodies the recombinant production of the protox enzyme, and methods for using recombinantly produced protox. The invention thus further embodies host cells, but especially cells slected from the group consisting of plant cells, animal cells, bacterial cells, yeast cells and insect cells, stably transformed with a recombinant DNA molecule comprising a suitable promoter functional in the respective host cell operably linked to a structural gene encoding an unmodified or modified eukaryotic protox enzyme, wherein said host cell is capable of expressing said DNA molecule.

The present invention further provides methods of using purified protox to screen for novel herbicides which affect the activity of protox, and to identify herbicide-resistant protox mutants.

In particular, the invention is directed to a method for assaying a chemical for the ability to inhibit the activity of a protox enzyme from a plant comprising

(a) combining said protox enzyme and protoporphyrinogen IX in a first reaction mixture under conditions in which said protox enzyme is capable of catalyzing the conversion of said protoporphyrinogen IX to protoporphyrin IX;

(b) combining said chemical, said protox enzyme and protoporphyrinogen IX in a second reaction mixture under the same conditions as in said first reaction mixture;

(c) exciting said first and said second reaction mixtures at about 395 to about 410 nM;

(d) comparing the flourescence of said first and said second reaction mixtures at about 622 to about 635 nM;

wherein said chemical is capable of inhibiting the activity of said protox enzyme if the flourescence of said second reaction mixture is significantly less than the flourescence

In a further embodiment of the invention a method is provided for identifying a modified protox enzyme resistant to a protox inhibitor present in a population of cells comprising the steps of

(a) culturing said population in the presence of said protox inhibitor in amounts which inhibit the unmodified form of said protox enzyme;

(b) selecting those cells from step (a) whose growth is not inhibited; and

(c) isolating and identifying the protox enzyme present in the cells selected from step (b).

Genes encoding altered protox can be used as selectable markers in plant cell transformation methods. The present invention thus further embodies a method of selecting plants, plant tissue or plant cells transformed with a transgene of interest from non-transformed plants, comprising the steps of:

(a) transforming a plant, plant tissue or plant cell with a transgene of interest capable of being expressed by the plant, and a gene encoding an altered protox resistant to a protox inhibitor;

(b) transferring the thus-transformed plants or plant cells to a medium comprising the protox inhibitor; and

(c) selecting the plants or plant cells which survive in the medium.

The present invention is further directed to probes and methods for detecting the presence and form of the protox gene and quantitating levels of protox transcripts in an organism. These methods may be used to diagnose disease conditions which are associated with an altered form of the protox enzyme or altered levels of expression of the protox enzyme.

In one aspect, the present invention is directed to an isolated DNA molecule which encodes a eukaryotic form of protoporphyrinogen oxidase (referred to herein as "protox"), the enzyme which catalyzes the oxidation of protoporphyrinogen IX to protoporphyrin IX. The DNA coding sequences and corresponding amino acid sequences for protox enzymes from *Arabidopsis thaliana* are provided as SEQ ID Nos

1-4 and 9-10. The DNA coding sequences and corresponding amino acid sequences for maize protox enzymes are provided as SEQ ID Nos 5-8.

Any desired eukaryotic DNA encoding the protox enzyme may be isolated according to the invention. One method taught for isolating a eukaryotic protox coding sequence is represented by Example 1. In this method cDNA clones encoding a protox enzyme are identified from a library of cDNA clones derived from the eukaryote of interest based on their ability to supply protox enzymatic activity to a mutant host organism deficient in this activity. Suitable host organisms for use in this method are those which can be used to screen cDNA expression libraries and for which mutants deficient in protox activity are either available or can be routinely generated. Such host organisms include, but are not limited to, *E. coli* (Sasarman *et al., J. Gen. Microbiol. 113:* 297 (1979)), *Salmonella typhimurium* (Xu *et al., J. Bacteriol. 174*: 3953 (1992)), and *Saccharomyces cerevisiae* (Camadro *et al. Biochem. Biophys. Res. Comm. 106:* 724 (1982)).

Alternatively, eukaryotic protox coding sequences may be isolated according to well known techniques based on their sequence homology to the *Arabidopsis thaliana* (SEQ ID Nos. 1,3 and 9) and *Zea mays* (SEQ ID Nos. 5 and 7) protox coding sequences taught by the present invention. In these techniques all or part of the known protox coding sequence is used as a probe which selectively hybridizes to other protox coding sequences present in population of cloned genomic DNA fragments or cDNA fragments (i.e. genomic or cDNA libraries) from a chosen organism. Such techniques include hybridization screening of plated DNA libraries (either plaques or colonies; see, *e.g.*. Sambrook *et al.*, Molecular Cloning , eds., Cold Spring Harbor Laboratory Press. (1989)) and amplification by PCR using oligonucleotide primers corresponding to sequence domains conserved among known protox amino acid sequences (see, *e.g.* Innis *et al.*, . PCR Protocols, a Guide to Methods and Applications eds., Academic Press (1990)). These methods are particularly well suited to the isolation of protox coding sequences from organisms related to the organism from which the probe sequence is derived. For example, application of these methods using the *Arabidopsis* or *Zea mays* coding sequence as a probe would be expected to be particularly well suited for the isolation of protox coding sequences from other plant species.

The isolated eukaryotic protox sequences taught by the present invention may be manipulated according to standard genetic engineering techniques to suit any desired purpose. For example, the entire protox sequence or portions thereof may be used as probes capable of specifically hybridizing to protox coding sequences and messenger RNAs. To achieve specific hybridization under a variety of conditions, such probes include sequences that are unique among protox coding sequences and are preferably at least 10 nucleotides in length, and most preferably at least 20 nucleotides in length. Such probes may be used to amplify and analyze protox coding sequences from a chosen organism via the well known process of polymerase chain reaction (PCR). This technique may be useful to isolate additional protox coding sequences from a desired organism or as a diagnostic assay to determine the presence of protox coding sequences in an organism and to associate altered coding sequences with particular adverse conditions such as autosomal dominant disorder in humans characterized by both neuropsychiatric symptoms and skin lesions, have decreased levels of protox activity (Brenner and Bloomer, *New Engl. J. Med. 302:* 765 (1980)).

Protox specific hybridization probes may also be used to map the location of the native eukaryotic protox gene(s) in the genome of a chosen organism using standard techniques based on the selective hybridization of the probe to genomic protox sequences. These techniques include, but are not limited to, identification of DNA polymorphisms identified or contained within the protox probe sequence, and use of such polymorphisms to follow segregation of the protox gene relative to other markers of known map position in a mapping population derived from self fertilization of a hybrid of two polymorphic parental lines (see e.g. Helentjaris *et al.*, *Plant Mol. Biol. 5:* 109 (1985). Sommer *et al. Biotechniques 12:*82 (1992); D'Ovidio *et al.*, *Plant Mol. Biol. 15:* 169 (1990)). While any eukaryotic protox sequence is contemplated to be useful as a probe for mapping protox genes from any eukaryotic organism, preferred probes are those protox sequences from organisms more closely related to the chosen organism, and most preferred probes are those protox sequences from the chosen organism. Mapping of protox genes in this manner is contemplated to be particularly useful in plants for breeding purposes. For instance, by knowing the genetic map position of a mutant protox gene that confers herbicide resistance, flanking DNA markers can be

identified from a reference genetic map (see, *e.g.*, Helentjaris, *Trends Genet. 3:* 217 (1987)). During introgression of the herbicide resistance trait into a new breeding line, these markers can then be used to monitor the extent of protox-linked flanking chromosomal DNA still present in the recurrent parent after each round of back-crossing.

Protox specific hybridization probes may also be used to quantitate levels of protox mRNA in an organism using standard techniques such as Northern blot analysis. This technique may be useful as a diagnostic assay to detect altered levels of protox expression that may be associated with particular adverse conditions such as autosomal dominant disorder in humans characterized by both neuropsychiatric symptoms and skin lesions, have decreased levels of protox activity (Brenner and Bloomer, *New Engl. J. Med. 302:* 765 (1980)).

For recombinant production of the enzyme in a host organism, the protox coding sequence may be inserted into an expression cassette designed for the chosen host and introduced into the host where it is recombinantly produced. The choice of specific regulatory sequences such as promoter, signal sequence, 5' and 3' untranslated sequences, and enhancer, is within the level of skill of the routineer in the art. The resultant molecule, containing the individual elements linked in proper reading frame, may be inserted into a vector capable of being transformed into the host cell. Suitable expression vectors and methods for recombinant production of proteins are well known for host organisms such as *E. coli* (see, *e.g.* Studier and Moffatt, *J. Mol. Biol. 189:* 113 (1986); Brosius, *DNA 8:* 759 (1989)), yeast (see, *e.g.*, Schneider and Guarente, *Meth. Enzymol. 194*: 373 (1991)) and insect cells (see, *e.g.*, Luckow and Summers, *Bio/Technol. 6:* 47 (1988)). Specific examples include plasmids such as pBluescript (Stratagene, La Jolla, CA), pFLAG (International Biotechnologies, Inc., New Haven, CT), pTrcHis (Invitrogen, La Jolla, CA), and baculovirus expression vectors, e.g., those derived from the genome of *Autographica californica* nuclear polyhedrosis virus (AcMNPV). A preferred baculovirus/insect system is pVl11392/Sf21 cells (Invitrogen, La Jolla, CA).

Recombinantly produced eukaryotic protox enzyme is useful for a variety of purposes. For example, it may be used to supply protox enzymatic activity *in vitro*. It

may also be used in an *in vitro* assay to screen known herbicidal chemicals whose target has not been identified to determine if they inhibit protox. Such an *in vitro* assay may also be used as a more general screen to identify chemicals which inhibit protox activity and which are therefore herbicide candidates. Alternatively, recombinantly produced protox enzyme may be used to further characterize its association with known inhibitors in order to rationally design new inhibitory herbicides as well as herbicide tolerant forms of the enzyme.

Typically, the inhibitory effect on protox is determined by measuring fluorescence at about 622 to 635 nm, after excitation at about 395 to 410 nM (see, *e.g.* Jacobs and Jacobs, *Enyzme 26:* 206 (1982); Sherman *et al.*, *Plant Physiol. 97:* 280 (1991)). This assay is based on the fact that protoporphyrin IX is a fluorescent pigment, and protoporphyrinogen IX is nonfluorescent. Protein extracts are prepared from selected subcellular fractions, *e.g.* etioplasts, mitochondria, microsomes, or plasma membrane, by differential centrifugation (see, *e.g.* Lee *et al.*, *Plant Physiol. 102*:881 (1993); Prado *et al*, *Plant Physiol. 65:* 956 (1979); Jackson and Moore, *in* Plant Organelles. Reid, ed., pp. 1-12; Jacobs and Jacobs, *Plant Physiol. 101:* 1181 (1993)). Protoporphyrinogen is prepared by reduction of protoporphyrin with a sodium amalgam as described by Jacobs and Jacobs (1982). Reactions mixtures typically consist of 100 mM Hepes (pH 7.5), 5 mM EDTA, 2 mM DTT, about 2 M protoporphyrinogen IX, and about 1 mg/mL protein extract. Inhibitor solutions in various concentrations, e.g. 1 mM, 100 uM, 10 uM, 1 uM, 100 nM, 10 nM, 1nM, 100pM, are added to the enzyme extract prior to the initiation of the enzyme reaction. Once the protein extract is added, fluorescence is monitored for several minutes, and the slope of the slope (reaction rate) is calculated from a region of linearity. IC_{50} is determined by comparing the slope of the inhibited reaction to a control reaction.

Another embodiment of the present invention involves the use of protox in an assay to identify inhibitor-resistant protox mutants. A typical assay is as follows:

(a) incubating a first sample of protox and its substrate, protoporphyrinogen IX, in the presence of a second sample comprising a protox inhibitor;

(b) measuring the enzymatic activity of the protox from step (a);

(c) incubating a first sample of a mutated protox and its substrate in the presence of a second sample comprising the same protox inhibitor;

(d) measuring the enzymatic activity of the mutated protox from step (c); and

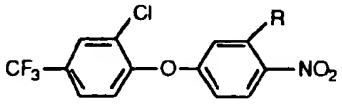
(e) comparing the enzymatic activity of the mutated protox with that provided by the unmutated protox.

The reaction mixture and the reaction conditions are the same as for the assay to identify inhibitors of protox (inhibitor assay) with the following modifications. First, a protox mutant, obtained as described above, is substituted in one of the reaction mixtures for the wild-type protox of the inhibitor assay. Second, an inhibitor of wild-type protox is present in both reaction mixtures. Third, mutated activity (enzyme activity in the presence of inhibitor and mutated protox) and unmutated activity (enzyme activity in the presence of inhibitor and wild-type protox) are compared to determine whether a significant increase in enzyme activity is observed in the mutated activity when compared to the unmutated activity. Mutated activity is any measure of enzymatic activity of the mutated protox enzyme while in the presence of a suitable substrate and the inhibitor. Unmutated activity is any measure of enzymatic activity of the wild-type protox enzyme while in the presence of a suitable substrate and the inhibitor. A significant increase is defined as an increase in enzymatic activity that is larger than the margin of error inherent in the measurement technique, preferably an increase by about 2-fold of the activity of the wild-type enzyme in the presence of the inhibitor, more preferably an increase by about 5-fold, most preferably an increase greater than by about 10-fold.

The herbicides that inhibit protox include many different structural classes of molecules (Duke *et al., Weed Sci. 39*: 465 (1991); Nandihalli *et al., Pesticide Biochem. Physiol. 43*: 193 (1992); Matringe *et al., FEBS Lett. 245*: 35 (1989); Yanase and Andoh, *Pesticide Biochem. Physiol. 35*: 70 (1989)), including the diphenylethers (e.g. acifluorifen, 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobezoic acid; its methyl ester; or oxyfluorfen, 2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluorobenzene)), oxidiazoles (e.g. oxidiazon, 3-[2,4-dichloro-5-(1-methylethoxy)phenyl]-5-(1,1-dimethylethyl)-1,3,4-oxadiazol-2-(3*H*)-one), cyclic imides (e.g. S-23142, *N*-(4-chloro-2-fluoro-5-propargyloxyphenyl)-3,4,5,6-tetrahydrophthalimide; chlorophthalim, *N*-(4-chlorophenyl)-

3,4,5,6-tetrahydrophthalimide), phenyl pyrazoles (e.g. TNPP-ethyl, ethyl 2-[1-(2,3,4-trichlorophenyl)-4-nitropyrazolyl-5-oxy]propionate; M&B 39279), pyridine derivatives (e.g. LS 82-556), and phenopylate and its *O*-phenylpyrrolidino- and piperidinocarbamate analogs.

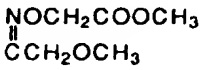
The diphenylethers of particular significance are those having the general formula



(Formula I)

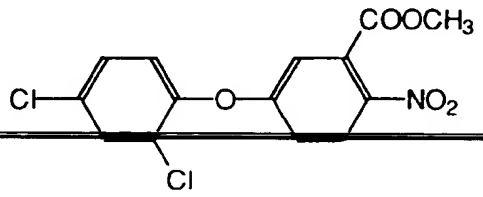
wherein R equals -COONa (Formula II), $-CONHSO_2CH_3$ (Formula III) or $-COOCH_2COOC_2H_5$ (Formula IV; *see* Maigrot *et al., Brighton Crop Protection Conference-Weeds:* 47-51 (1989)).

Additional diphenylethers of interest are those where R equals:



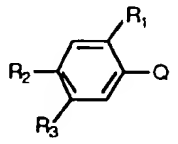
(Formula IVa; *see* Hayashi *et al., Brighton Crop Protection Conference-Weeds:* 53-58 (1989)).

An additional diphenylether of interest is one having the formula:



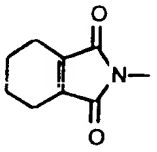
(Formula IVb; bifenox, *see* Dest *et al., Proc. Northeast Weed Sci. Conf. 27:* 31 (1973)).

Also of significance are the class of herbicides known as imides, having the general formula

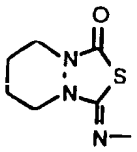


(Formula V)

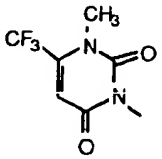
wherein Q equals



OR



OR



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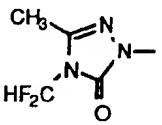
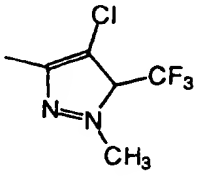
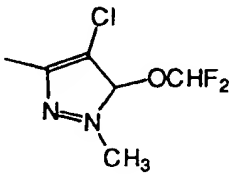


(Formula VI) (Formula VII) (Formula VIII) (Formula IX)



OR

(Formula IXa)

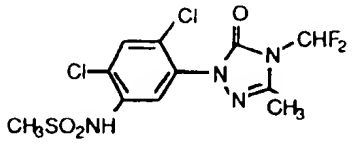


OR

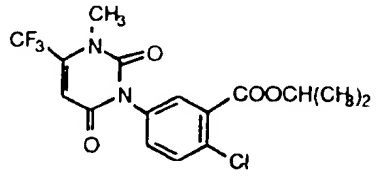
(Formula Ixb)

(*see* Hemper *et al.* (1995) *in* "Proceedings of the Eighth International Congress of Pesticide Chemistry", Ragdale *et al.*, eds., Amer. Chem. Soc, Washington, D.C., pp.42-48 (1994)).

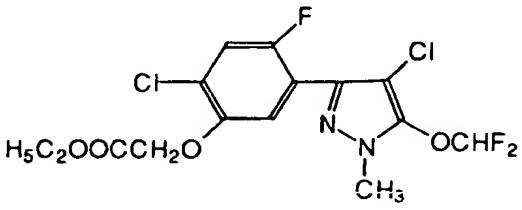
and R_1 equals H, Cl or F, R_2 equals Cl and R_3 is an optimally substituted ether, thioether, ester, amino or alkyl group. Alternatively, R_2 and R_3 together may form a 5 or 6 membered heterocyclic ring. Examples of imide herbicides of particular interest are



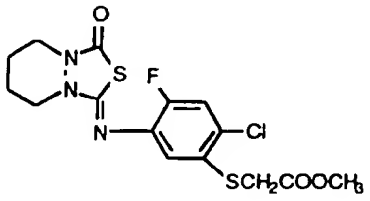
(Formula X)



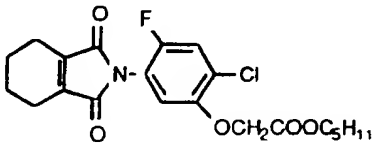
(Formula XI)



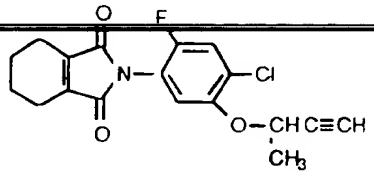
(Formula XII)
(*see* Miura *et al.*, *Brighton Crop Protection Conference-Weeds:* 35-40 (1993))



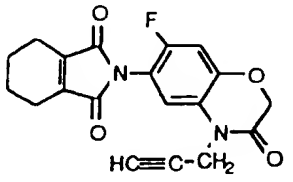
(Formula XIII)



(Formula XIV)



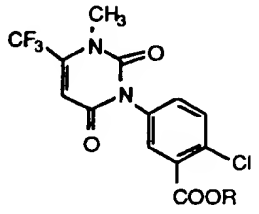
(Formula XV)



(Formula XVI)

The herbicidal activity of the above compounds is described in the *Proceedings of the 1991 Brighton Crop Protection Conference, Weeds* (British Crop Protection Council) (Formulae X and XVI), *Proceedings of the 1993 Brighton Crop Protection Conference, Weeds* (British Crop Protection Council) (Formulae XII and XIII), U.S. Patent No. 4,746,352 (Formula XI) and *Abstracts of the Weed Science Society of America* vol. 33, pg. 9 (1993)(Formula XIV).

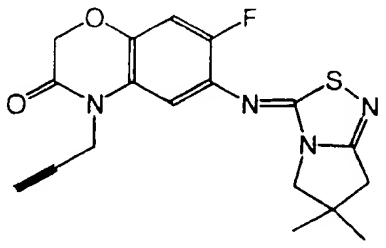
The most preferred imide herbicides are those classified as aryluracils and having the general formula



(Formula XVII)

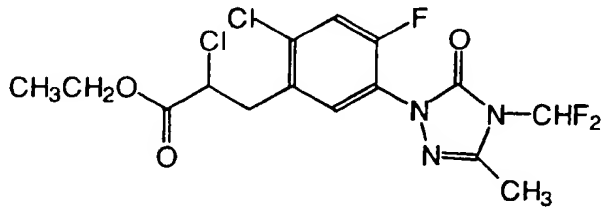
wherein R signifies the group (C_{2-6}-alkenyloxy)carbonyl-C_{1-4}-alkyl, as disclosed in U.S. Patent No. 5,183,492, herein incorporated by reference.

Also of significance are herbicides having the general formula:



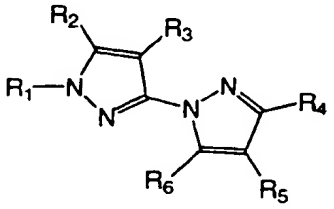
(Formula XVIII: thiadiazimin)

(*see* Weiler *et al., Brighton Crop Protection Conference-Weeds*, pp. 29-34 (1993));



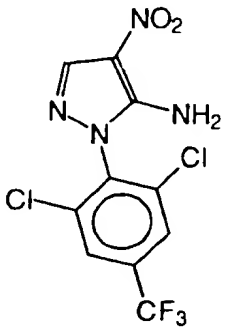
(Formula XIX; carfentrazone)
(*see* Van Saun *et al., Brighton Crop Protection Conference-Weeds:* pp. 19-22 (1993));

N-substituted pyrazoles of the general formula:



(Formula XX)
(*see* international patent publications WO 94/08999, WO 93/10100, and U. S. Patent No. 5,405,829 assigned to Schering);

N-phenylpyrazoles, such as:



(Formula XXI; nipyraclofen)
(*see* page 621 of "The Pesticide Manual", 9th ed., ed. by C.R. Worthing, British Crop Protection Council, Surrey (1991));

and 3-substituted-2-aryl-4,5,6,7-tetrahydroindazoles (Lyga *et al. Pesticide Sci. 42*:29-36 (1994)).

Levels of herbicide which normally are inhibitory to the activity of protox include application rates known in the art, and which depend partly on external factors such as environment, time and method of application. For example, in the case of the imide herbicides represented by Formulae V through IX, and more particularly those represented by Formulae X through XVII, the application rates range from 0.0001 to 10 kg/ha, preferably from 0.005 to 2 kg/ha. This dosage rate or concentration of herbicide may be different, depending on the desired action and particular compound used, and can be determined by methods known in the art.

The present invention is further directed to plants, plant tissue and plant seeds tolerant to herbicides that inhibit the naturally occurring protox activity in these plants, wherein the tolerance is conferred by an altered protox enzyme activity. Representative plants include any plants to which these herbicides are applied for their normally intended purpose. Preferred are agronomically important crops, i.e., angiosperms and gymnosperms significant as cotton, soya, rape, sugar beet, maize, rice, wheat, barley, oats, rye, sorghum, millet, turf, forage, turf grasses and the like.

By "altered protox enzyme activity" is meant a protox enzymatic activity different from that which naturally occurs in a plant (i.e. protox activity which occurs naturally in the absence of direct or indirect manipulation of such activity by man) which is resistant

to herbicides that inhibit the naturally occurring activity. Altered protox enzyme activity may be conferred upon a plant according to the invention by increasing expression of wild-type, herbicide-sensitive protox, expressing an altered, herbicide-tolerant eukaryotic protox enzyme in the plant, expressing an unmodified or modified bacterial form of the protox enzyme which is herbicide resistant in the plant, or by a combination of these techniques.

Achieving altered protox enzyme activity through increased expression results in a level of protox in the plant cell at least sufficient to overcome growth inhibition caused by the herbicide. The level of expressed protox generally is at least two times, preferably five times, and more preferably at least ten times the natively expressed amount. Increased expression may be due to multiple copies of a wild-type protox gene; multiple occurrences of the protox coding sequence within the protox gene (i.e. gene amplification) or a mutation in the non-coding, regulatory sequence of the endogenous protox gene in the plant cell. Plants containing such altered protox enzyme activity can be obtained by direct selection in plants. This method is known in the art. *See, e.g.* Somers et al. in U.S. 5,162,602, and Anderson *et al.* in U.S. 4,761,373, and references cited therein. These plants also may be obtained via genetic engineering techniques known in the art.

Increased expression of herbicide-sensitive protox also can be accomplished by stably transforming a plant cell with a recombinant or chimeric DNA molecule comprising a promoter capable of driving expression of an associated structural gene in a plant cell, linked to a homologous or heterologous structural gene encoding protox. By "homologous," it is meant that the protox gene is isolated from an organism taxonomically identical to the target plant cell. By "heterologous," it is meant that the protox gene is obtained from an organism taxonomically distinct from the target plant cell. Homologous protox genes can be obtained by complementing a bacterial or yeast auxotrophic mutant with a cDNA expression library from the target plant. *See, e.g.* Example 1 and Snustad *et al, Genetics 120*:1111-1114 (1988) (maize glutamine synthase); Delauney *et al., Mol. Genet. 221*:299-305 (1990) (soybean -pyrroline -5-carboxylate reductase); Frisch *et al., Mol. Gen. Genet. 228*:287-293(1991) (maize dihydrodipicolinate synthase); Eller *et al., Plant Mol. Biol. 18*:557-566 (1992) (rape

chloroplast 3-isopropylmalate dehydrogenase); *Proc. Natl. Acad. Sci, USA 88*:1731-1735 (1991); Minet *et al., Plant J. 2*:417-422 (1992) (dihydroorotate dehydrogenase) and references cited therein. Other known methods include screening genomic or cDNA libraries of higher plants, for example, for sequences that cross-hybridize with specific nucleic acid probes, or by screening expression libraries for the production of protox enzymes that cross-react with specific antibody probes. A preferred method involves complementing an *E. coli hemG* auxotrophic mutant with a maize or *Arabidopsis thaliana* cDNA library.

Examples of promoters capable of functioning in plants or plant cells, i.e., those capable of driving expression of the associated structural genes such as protox in plant cells, include the cauliflower mosaic virus (CaMV) 19S or 35S promoters and CaMV double promoters; nopaline synthase promoters; pathogenesis-related (PR) protein promoters; small subunit of ribulose bisphosphate carboxylase (ssuRUBISCO) promoters, and the like. Preferred are the rice actin promoter (McElroy *et al., Mol. Gen. Genet. 231*: 150 (1991)), maize ubiquitin promoter (EP 0 342 926; Taylor *et al., Plant Cell Rep.12:* 491 (1993)), and the Pr-1 promoter from tobacco, *Arabidopsis*, or maize (see International Patent Application No. PCT/IB95/00002 to Ryals *et al.*, incorporated by reference herein in its entirety). Also preferred are the 35S promoter and an enhanced or double 35S promoter such as that described in Kay *et al., Science 236:* 1299-1302 (1987) and the double 35S promoter cloned into pCGN2113, deposited as ATCC 40587, which are disclosed in EP-A 0 392 225, the relevant disclosures of which are herein incorporated by reference in their entirety. The promoters themselves may be modified to manipulate promoter strength to increase protox expression, in accordance with art-recognized procedures.

Signal or transit peptides may be fused to the protox coding sequence in the chimeric DNA constructs of the invention to direct transport of the expressed protox enzyme to the desired site of action. Examples of signal peptides include those natively linked to the plant pathogenesis-related proteins, e.g. PR-1, PR-2, and the like. *See, e.g.*, Payne *et al., Plant Mol. Biol. 11*:89-94 (1988). Examples of transit peptides include the chloroplast transit peptides such as those described in Von Heijne *et al., Plant Mol. Biol. Rep. 9*:104-126 (1991); *Mazur et al., Plant Physiol. 85*: 1110 (1987);

Vorst *et al., Gene 65*: 59 (1988), and mitochondrial transit peptides such as those described in Boutry *et al., Nature 328*:340-342 (1987). Chloroplast and mitochondrial transit peptides are contemplated to be particularly useful with the present invention as protox enzymatic activity typically occurs within the mitochondria and chloroplast. Most preferred for use are chloroplast transit peptides as inhibition of the protox enzymatic activity in the chloroplasts is contemplated to be the primary basis for the action of protox-inhibiting herbicides (Witkowski and Halling, *Plant Physiol. 87:* 632 (1988); Lehnen *et al., Pestic. Biochem. Physiol. 37*: 239 (1990); Duke *et al., Weed Sci. 39*: 465 (1991)). Also included are sequences that result in localization of the encoded protein to various cellular compartments such as the vacuole. *See, for example*, Neuhaus *et al., Proc. Natl. Acad. Sci. USA 88:* 10362-10366 (1991) and Chrispeels, *Ann. Rev. Plant Physiol. Plant Mol. Biol. 42*: 21-53 (1991). The relevant disclosures of these publications are incorporated herein by reference in their entirety.

The chimeric DNA construct(s) of the invention may contain multiple copies of a promoter or multiple copies of the protox structural genes. In addition, the construct(s) may include coding sequences for markers and coding sequences for other peptides such as signal or transit peptides, each in proper reading frame with the other functional elements in the DNA molecule. The preparation of such constructs are within the ordinary level of skill in the art.

Useful markers include peptides providing herbicide, antibiotic or drug resistance, such as, for example, resistance to hygromycin, kanamycin, G418, gentamycin, lincomycin, methotrexate, glyphosate, phosphinothricin, or the like. These markers can be used to select cells transformed with the chimeric DNA constructs of the invention from untransformed cells. Other useful markers are peptidic enzymes which can be easily detected by a visible reaction, for example a color reaction, for example luciferase, ß-glucuronidase, or ß-galactosidase.

Altered protox enzyme activity may also be achieved through the generation or identification of modified forms of the isolated eukaryotic protox coding sequence having at least one amino acid substitution, addition or deletion which encode an altered protox enzyme resistant to a herbicide that inhibits the unaltered, naturally occuring form (i.e. forms which occur naturally in a eukaryotic organism without being

manipulated, either directly *via* recombinant DNA methodology or indirectly *via* selective breeding, etc., by man). Genes encoding such enzymes can be obtained by numerous strategies known in the art. A first general strategy involves direct or indirect mutagenesis procedures on microbes. For instance, a genetically manipulable microbe, e.g. *E. coli* or *S. cerevisiae*, may be subjected to random mutagenesis *in vivo*, with, for example UV light or ethyl or methyl methane sulfonate. Mutagenesis procedures are described, for example in Miller, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1972); Davis *et al.*, Advanced Bacterial Genetics. Cold Spring Harbor Laboratory. Cold Spring Harbor. NY (1980); Sherman *et al.*, Methods in Yeast Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1983); and U.S. Patent No. 4,975,374 (Goodman *et al*). The microbe selected for mutagenesis contains a normally herbicide sensitive eukaryotic protox gene and is dependent upon the protox activity conferred by this gene. The mutagenized cells are grown in the presence of the herbicide at concentrations which inhibit the unmodified protox enzyme. Colonies of the mutagenized microbe that grow better than the unmutagenized microbe in the presence of the inhibitor (i.e. exhibit resistance to the inhibitor) are selected for further analysis. The protox genes from these colonies are isolated, either by cloning or by polymerase chain reaction amplification, and their sequences elucidated. Sequences encoding an altered protox enzyme are then cloned back into the microbe to confirm their ability to confer inhibitor resistance.

A second method of obtaining mutant herbicide-resistant alleles of the eukaryotic protox enzyme involves direct selection in plants. For example, the effect of a protox inhibitor such those as described above, on the growth inhibition of plants such as *Arabidopsis*, soybean, or maize may be determined by plating seeds sterilized by art-recognized methods on plates on a simple minimal salts medium containing increasing concentrations of the inhibitor. Such concentrations are in the range of 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 110, 300, 1000 and 3000 parts per million (ppm). The lowest dose at which significant growth inhibition can be reproducibly detected is used for subsequent experiments.

Mutagenesis of plant material may be utilized to increase the frequency at which resistant alleles occur in the selected population. Mutagenized seed material can be

derived from a variety of sources, including chemical or physical mutagenesis or seeds, or chemical or physical mutagenesis or pollen (Neuffer, *In* Maize for Biological Research. Sheridan, ed. Univ.Press, Grand Forks, ND., pp. 61-64 (1982)), which is then used to fertilize plants and the resulting M_1 mutant seeds collected. Typically, for *Arabidopsis*, M_2 seeds (Lehle Seeds, Tucson, AZ), i.e. progeny seeds of plants grown from seeds mutagenized with chemicals, such as ethyl methane sulfonate, or with physical agents, such as gamma rays or fast neutrons, are plated at densities of up to 10,000 seeds/plate (10 cm diameter) on minimal salts medium containing an appropriate concentration of inhibitor to select for resistance. Seedlings that continue to grow and remain green 7-21 days after plating are transplanted to soil and grown to maturity and seed set. Progeny of these seeds are tested for resistance to the herbicide. If the resistance trait is dominant, plants whose seed segregate 3:1::resistant:sensitive are presumed to have been heterozygous for the resistance at the M_2 generation. Plants that give rise to all resistant seed are presumed to have been homozygous for the resistance at the M_2 generation. Such mutagenesis on intact seeds and screening of their M2 progeny seed can also be carried out on other species, for instance soybean (*see,e.g.* U.S. Pat. No. 5,084,082 (Sebastian)). Mutant seeds to be screened for herbicide tolerance can also be obtained as a result of fertilization with pollen mutagenized by chemical or physical means.

Two approaches can be taken to confirm that the genetic basis of the resistance is an altered protox gene. First, alleles of the protox gene from plants exhibiting resistance to the inhibitor can be isolated using PCR with primers based either upon conserved regions in the *Arabidopsis* and maize protox cDNA sequences shown in SEQ ID NOS:1,3,5,7 below or, more preferably, based upon the unaltered protox gene sequences from the plant used to generate resistant alleles. After sequencing the alleles to determine the presence of mutations in the coding sequence, the alleles can be tested for their ability to confer resistance to the inhibitor on plants into which the putative resistance-conferring alleles have been transformed. These plants can be either *Arabidopsis* plants or any other plant whose growth is susceptible to the inhibitors. Second, the protox genes can be mapped relative to known restriction fragment length polymorphisms (RFLPs) (*See, for example*, Chang *et al. Proc. Natl.*

Acad, Sci, USA 85:6856-6860 (1988); Nam *et al., Plant Cell 1*:699-705 (1989). The resistance trait can be independently mapped using the same markers. If resistance is due to a mutation in that protox gene, the resistance trait will map to a position indistinguishable from the position of a protox gene.

A third method of obtaining herbicide-resistant alleles of protox is by selection in plant cell cultures. Explants of plant tissue, *e.g.* embryos, leaf disks, etc. or actively growing callus or suspension cultures of a plant of interest are grown on defined medium lacking heme in the presence of increasing concentrations of the inhibitory herbicide or an analogous inhibitor suitable for use in a laboratory environment. Varying degrees of growth are recorded in different cultures. In certain cultures, fast-growing variant colonies arise that continue to grow even in the presence of normally inhibitory concentrations of inhibitor. The frequency with which such faster-growing variants occur can be increased by treatment with a chemical or physical mutagen before exposing the tissues or cells to the herbicide. Putative resistance-conferring alleles of the protox gene are isolated and tested as described in the foregoing paragraphs. Those alleles identified as conferring herbicide resistance may then be engineered for optimal expression and transformed into the plant. Alternatively, plants can be regenerated from the tissue or cell cultures containing these alleles.

A fourth method involves mutagenesis of wild-type, herbicide sensitive protox genes in bacteria or yeast, followed by culturing the microbe on medium that lacks heme, but which contains inhibitory concentrations of the inhibitor and then selecting those colonies that grow in the presence of the inhibitor. More specifically, a plant cDNA, such as the *Arabidopsis* or maize cDNA encoding protox is cloned into a microbe that otherwise lacks protox activity. Examples of such microbes include *E. coli*, *S. typhimurium*, and *S. cerevisiae* auxotrophic mutants, including *E. coli* strain SASX38 (Sasarman *et al., J. Gen. Microbiol. 113:* 297 (1979), *S. typhimurium* strain TE2483 or TT13680 (Xu *et al., J. Bacteriol. 174:* 3953 (1992)), and the *hem14-1* yeast mutant (Camadro *et al., Biochem. Biophys. Res. Comm. 106*: 724 (1982)). The transformed microbe is then subjected to *in vivo* mutagenesis such as described immediately above, or to *in vitro* mutagenesis by any of several chemical or enzymatic methods known in

the art, e.g. sodium bisulfite (Shortle *et al., Methods Enzymol. 100:*457-468 (1983); methoxylamine (Kadonaga *et al., Nucleic Acids Res. 13:*1733-1745 (1985); oligonucleotide-directed saturation mutagenesis (Hutchinson *et al., Proc. Natl. Acad. Sci. USA, 83:*710-714 (1986); or various polymerase misincorporation strategies (*see, e.g.* Shortle *et al., Proc. Natl. Acad. Sci. USA, 79:*1588-1592 (1982); Shiraishi *et al., Gene 64:*313-319 (1988); and Leung *et al., Technique 1:*11-15 (1989). Colonies that grow in the presence of normally inhibitory concentrations of inhibitor are picked and purified by repeated restreaking. Their plasmids are purified and tested for the ability to confer resistance to the inhibitor by retransforming them into the protox-lacking microbe. The DNA sequences of protox cDNA inserts from plasmids that pass this test are then determined.

Once a herbicide resistant protox allele is identified, it may be genetically engineered for optimal expression in a crop plant. This may include altering the coding sequence of the resistance allele for optimal expression in the crop species of interest. Methods for modifying coding sequences to achieve optimal expression in a particular crop species are well known (see, *e.g.* Perlak *et al., Proc. Natl. Acad. Sci. USA 88:* 3324 (1991); Koziel *et al., Bio/technol. 11:* 194 (1993)). Genetically engineering the protox allele for optimal expression may also include operably linking the appropriate regulatory sequences (i.e. promoter, signal sequence, transcriptional terminators). Preferred promoters will be those which confer high level constitutive expression or, more preferably, those which confer specific high level expression in the tissues susceptible to damage by the herbicide.

The recombinant DNA molecules can be introduced into the plant cell in a number of art-recognized ways. Those skilled in the art will appreciate that the choice of method might depend on the type of plant, i.e. monocot or dicot, targeted for transformation. Suitable methods of transforming plant cells include microinjection (Crossway *et al., BioTechniques 4:*320-334 (1986)), electroporation (Riggs *et al, Proc. Natl. Acad. Sci. USA 83:*5602-5606 (1986), *Agrobacterium* mediated transformation (Hinchee *et al., Biotechnology 6:*915-921 (1988)), direct gene transfer (Paszkowski *et al., EMBO J. 3:*2717-2722 (1984)), and ballistic particle acceleration using devices available from Agracetus, Inc., Madison, Wisconsin and Dupont, Inc., Wilmington, Delaware (*see, for*

example, Sanford *et al.*, U.S. Patent 4,945,050; and McCabe *et al.*, *Biotechnology 6*:923-926 (1988)). Also see, Weissinger *et al.*, *Annual Rev. Genet. 22*:421-477 (1988); Sanford *et al.*, *Particulate Science and Technology 5*:27-37 (1987)(onion); Christou *et al.*, *Plant Physiol. 87*:671-674 (1988)(soybean); McCabe *et al.*, *Bio/Technology 6*:923-926 (1988)(soybean); Datta *et al.*, *Bio/Technology 8*:736-740 (1990)(rice); Klein *et al.*, *Proc. Natl. Acad. Sci. USA, 85*:4305-4309 (1988)(maize); Klein *et al.*, *Bio/Technology 6*:559-563 (1988)(maize); Klein *et al.*, *Plant Physiol. 91*:440-444 (1988)(maize); Fromm *et al.*, *Bio/Technology 8*:833-839 (1990); and Gordon-Kamm *et al.*, *Plant Cell 2*:603-618 (1990)(maize).

Further comprised within the scope of the present invention are transgenic plants, in particular transgenic fertile plants transformed by means of the aforedescribed processes and their asexual and/or sexual progeny, which still are resistant or at least tolerant to inhibition by a herbicide at levels which normally are inhibitory to the naturally occurring protox activity in the plant. Very especially preferred are hybrid plants which are resistant or at least tolerant to inhibition by a herbicide at levels which normally are inhibitory to the naturally occurring protox activity in the plant.

The transgenic plant according to the invention may be a dicotyledonous or a monocotyledonous plant. Preferred are monocotyledonous plants of the *Graminaceae* family involving *Lolium*, *Zea*, *Triticum*, *Triticale*, *Sorghum*, *Saccharum*, *Bromus*, *Oryzae*, *Avena*, *Hordeum*, *Secale* and *Setaria* plants.

Especially preferred are transgenic maize, wheat, barley, sorghum, rye, oats, turf grasses and rice.

Among the dicotyledonous plants soybean, cotton, tobacco, sugar beet, oilseed rape, and sunflower are especially preferred herein.

The expression 'progeny' is understood to embrace both, "asexually" and "sexually" generated progeny of transgenic plants. This definition is also meant to include all mutants and variants obtainable by means of known processes, such as for example cell fusion or mutant selection and which still exhibit the characteristic properties of the initial transformed plant, together with all crossing and fusion products of the transformed plant material.

Another object of the invention concerns the proliferation material of transgenic plants.

The proliferation material of transgenic plants is defined relative to the invention as any plant material that may be propagated sexually or asexually *in vivo* or *in vitro*. Particularly preferred within the scope of the present invention are protoplasts, cells, calli, tissues, organs, seeds, embryos, pollen, egg cells, zygotes, together with any other propagating material obtained from transgenic plants.

Parts of plants, such as for example flowers, stems, fruits, leaves, roots originating in transgenic plants or their progeny previously transformed by means of the process of the invention and therefore consisting at least in part of transgenic cells, are also an object of the present invention.

Before the plant propagation material [fruit, tuber, grains, seed], but expecially seed is sold as a commerical product, it is customarily treated with a protectant coating comprising herbicides, insecticides, fungicides, bactericides, nematicides, molluscicides or mixtures of several of these preparations, if desired together with further carriers, surfactants or application-promoting adjuvants customarily employed in the art of formulation to provide protection against damage caused by bacterial, fungal or animal pests.

In order to treat the seed, the protectant coating may be applied to the seeds either by impregnating the tubers or grains with a liquid formulation or by coating them with a combined wet or dry formulation. In addition, in special cases, other methods of application to plants are possible, eg treatment directed at the buds or the fruit.

The plant seed according to the invention comprising a DNA sequence encoding a protein from a eukaryote having protoporphyrinogen oxidase (protox) activity according to the invention may be treated with a seed protectant coating comprising a seed treatment compound, such as, for example, captan, carboxin, thiram (TMTD®), methalaxyl (Apron®) and pirimiphos-methyl (Actellic®) and others that are commonly used in seed treatment.

It is thus a further object of the present invention to provide plant propagation material for cultivated plants, but especially plant seed that is treated with an seed protectant coating customarily used in seed treatment.

Where a herbicide resistant protox allele is obtained via direct selection in a crop plant or plant cell culture from which a crop plant can be regenerated, it may be moved into commercial varieties using traditional breeding techniques to develop a herbicide tolerant crop without the need for genetically engineering the allele and transforming it into the plant. Alternatively, the herbicide resistant allele may be isolated, genetically engineered for optimal expression and then transformed into the desired variety.

Genes encoding altered protox resistant to a protox inhibitor can also be used as selectable markers in plant cell transformation methods. For example, plants, plant tissue or plant cells transformed with a transgene can also be transformed with a gene encoding an altered protox capable of being expressed by the plant. The thus-transformed cells are transferred to medium containing the protox inhibitor wherein only the transformed cells will survive. Protox inhibitors contemplated to be particularly useful as selective agents are the diphenylethers {e.g. acifluorfen, 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobezoic acid; its methyl ester; or oxyfluorfen, 2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluorobenzene)}, oxidiazoles, (e.g. oxidiazon, 3-[2,4-dichloro-5-(1-methylethoxy)phenyl]-5-(1,1-dimethylethyl)-1,3,4-oxadiazol-2-(3*H*)-one), cyclic imides (e.g. S-23142, *N*-(4-chloro-2-fluoro-5-propargyloxyphenyl)-3,4,5,6-tetrahydrophthalimide; chlorophthalim, *N*-(4-chlorophenyl)-3,4,5,6-tetrahydrophthalimide), phenyl pyrazoles (e.g. TNPP-ethyl, ethyl 2-[1-(2,3,4-trichlorophenyl)-4-nitropyrazolyl-5-oxy]propionate; M&B 39279), pyridine derivatives (e.g. LS 82-556), and phenopylate and its *O*-phenylpyrrolidino- and piperidinocarbamate analogs. The method is applicable to any plant cell capable of being transformed with an altered protox-encoding gene, and can be used with any transgene of interest. Expression of the transgene and the protox gene can be driven by the same promoter functional on plant cells, or by separate promoters.

The invention will be further described by reference to the following detailed examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified.

DEPOSITS

The following vector molecules have been deposited with Agricultural Research Service, Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 North University Street, Peoria, Illinois 61604, U.S.A on the dates indicated below:

Protox-1, in the pBluescript SK vector, was deposited April 5, 1994 as pWDC-2 (#B-21238).

Protox-2, in the pFL61 vector, was deposited April 5, 1994 as pWDC-1 (NRRL #B-21237).

MzProtox-1, in the pBluescript SK vector, deposited May 20, 1994 as pWDC-4 with the NRRL (#B-21260), shown in SEQ ID NO:5.

MzProtox-1, in the pBluescript SK vector, redeposited July 11, 1994 as pWDC-4 with the NRRL (#B-21260N), shown in SEQ ID NO:5.

MzProtox-2, in the pBluescript SK vector, deposited May 20, 1994 as pWDC-3 with the NRRL (#B-21259), shown in SEQ ID NO:7.

Protox-3, in the pFL61 vector, was deposited June 10, 1994 as pWDC-5 (NRRL #B-21280).

pMzC-1Val, in the pBluescript SK vector, was deposited on September 30, 1994 under the designation pWDC-8 and given the deposit designation NRRL #21340.

pAraC-2Cys, in the pFL61 vector, was deposited on November 14, 1994 under the designation pWDC-7 and given the deposit designation NRRL #21339N.

EXAMPLES

Standard recombinant DNA and molecular cloning techniques used here are well known in the art and are described by T. Maniatis, E. F. Fritsch and J. Sambrook, Molecular Cloning - A Laboratory manual, Cold Spring Harbor laboratory, Cold Spring

Harbor, NY (1982) and by T.J. Silhavy, M.L. Berman, and L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984).

EXAMPLE 1: Isolation of Arabidopsis cDNAs encoding protox genes by functional complementation of an E. coli mutant.

An *Arabidopsis thaliana* (Landsberg) cDNA library in the plasmid vector pFL61 (Minet *et al.*, *Plant J. 2*:417-422 (1992)) was obtained and amplified. A second *Arabidopsis* (Columbia) cDNA library in the UniZap lambda vector (Stratagene) was purchased and amplified as pBluescript plasmids by mass *in vivo* excision of the phage stock. The *E. coli hemG* mutant SASX38 (Sasarman *et al.*, *J. Gen. Microbiol. 113:* 297 (1979)) was obtained and maintained on L media containing 20mg/ml hematin (United States Biochemicals). The plasmid libraries were transformed into SASX38 by electroporation using the Bio-Rad Gene Pulser and the manufacturer's conditions. The cells were plated on L agar containing 100mg/ml ampicillin at a density of approximately 500,000 transformants/10 cm plate. The cells were incubated at 37° C for 40 hours in low light and selected for the ability to grow without the addition of exogenous heme. Heme prototrophs were recovered at a frequency of $400/10^7$ from the pFL61 library and at a frequency of $2/10^7$ from the pBluescript library. Plasmid DNA was isolated from 24 colonies for sequence analysis. Each of the 24 was retransformed into SASX38 to verify ability to complement.

Sequence analysis revealed two classes of putative protox clones. Nine were of the type designated "Protox-1." Each was derived from the same gene, and two were full-length clones. The cDNA is 1719bp in length and encodes a protein of molecular weight 57.7 kDa. The N-terminal peptide sequence has features characteristic of a chloroplast transit peptide of approximately 60 amino acids. A database search with the GAP program (Deveraux *et al.*, *Nucleic Acids Res. 12*:387-395 (1984) reveals homology with the *B. subtilis hemY* (protox) protein (Hansson and Hederstedt 1992, Dailey *et al.*, *J. Biol. Chem. 269*: 813 (1994)). The two proteins are 53% similar, 31% identical with regions of high homology, including the proposed dinucleotide binding domain of the *hemY* protein (Dailey *et al.*, *J. Biol. Chem. 269*: 813 (1994)).

The other 15 cDNA clones were of the type designated "Protox-2". These also

length and encodes a protein of molecular weight 55.6kD. The amino terminus is somewhat characteristic of a mitochondrial transit peptide. The Protox-2 protein has limited homology to Protox-1 (53% similar, 28% identical) and to the *B. subtilis* protox (50% similar, 27% identical).

Protox-1, in the pBluescript SK vector, was deposited April 5, 1994 as pWDC-2 (NRRL #B-21238).

Protox-2, in the pFL61 vector, was deposited April 5, 1994 as pWDC-1 (NRRL #B-21237).

The Arabidopsis cDNA encoding protox-1 contained in pWDC-2 and protox-2 contained in pWDC-1 are set forth in SEQ ID NOS:1 and 3, respectively, below.

EXAMPLE 2: Isolation of Maize cDNAs encoding protox genes by functional complementation of an E. coli mutant.

A *Zea Mays* (B73 inbred) cDNA library in lambda UniZap was purchased from Stratagene and converted to a pBluescript library by mass *in vivo* excision. A second custom-made UniZap maize cDNA library was purchased from Clontech, and similarly converted to pBluescript plasmids. Selection for functional protox genes from maize was just as described for the *Arabidopsis* libraries above in Example 1.

Two heme prototrophs in 10^7 transformants were isolated from the Stratagene library, shown to recomplement and sequenced. These cDNAs were identical and proved to be homologs of *Arabidopsis* Protox-1. This maize clone, designated MzProtox-1, is incomplete. The cDNA is 1698bp in length and codes only for the putative mature protox enzyme; there is no transit peptide sequence and no initiating methionine codon. The gene is 68% identical to Arab Protox-1 at the nucleotide level and 76% identical (87% similar) at the amino acid level (shown in Table 1).

A single heme prototroph in 10^7 transformants was obtained from the Clontech library, shown to recomplement, and sequenced. The cDNA appears to be complete, is 2061 bp in length and encodes a protein of 59 kDa. This clone is a maize homolog of *Arabidopsis* Protox-2 and is designated MzProtox-2. The gene is 58% identical to Arab Protox-2 at the nucleotide level and 58% identical (76% similar) at the amino acid level (shown in Table 2). The maize clone has an N-terminal sequence that is 30 amino acids longer than the Arabidopsis clone. As with the Arabidopsis clones, homology

between the two maize protox genes is quite low, with only 31% identity between the two protein sequences.

MzProtox-1, in the pBluescript SK vector, deposited May 20, 1994 as pWDC-4 with the NRRL (#B-21260), shown in SEQ ID NO:5.

MzProtox-1, in the pBluescript SK vector, redeposited July 11, 1994 as pWDC-4 with the NRRL (#B-21260N), shown in SEQ ID NO:5.

MzProtox-2, in the pBluescript SK vector, deposited May 20, 1994 as pWDC-3 with the NRRL (#B-21259), shown in SEQ ID NO:7.

EXAMPLE 3: Isolation of additional protox genes based on sequence homology to known protox coding sequences

A phage or plasmid library is plated at a density of approximately 10,000 plaques on a 10 cm Petri dish, and filter lifts of the plaques are made after overnight growth of the plants at 37 C. The plaque lifts are probed with one of the cDNAs set forth in SEQ ID NOS:1, 3, 5 or 7, labeled with 32P-dCTP by the random priming method by means of a PrimeTime kit (International Biotechnologies, Inc., New Haven, CT). Hybridization conditions are 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO4 pH 7.0, 1 mM EDTA at 50 C. After hybridization overnight, the filters are washed with 2X SSC, 1% SDS. Positively hybridizing plaques are detected by autoradiography. After purification to single plaques, cDNA inserts are isolated, and their sequences determined by the chain termination method using dideoxy terminators labeled with fluorescent dyes (Applied Biosystems, Inc., Foster City, CA).

The standard experimental protocol described above can be used by one of skill in the art to obtain protox genes sequentially homologous to the known protox coding sequences from any other eukaryote, particularly other higher plant species.

An alignment of the predicted amino acid sequences of the respective proteins encoded by the sequences shown in SEQ ID NOS: 2 and 6 are set forth in Table 1. An alignment of the predicted amino acid sequences of the respective proteins encoded by the sequences shown in SEQ ID NOS: 4 and 8 are set forth in Table 2.

TABLE 1

Comparison of the Arabidopsis (SEQ ID No. 2) and Maize (SEQ ID No. 6) Protox-1 Amino Acid Sequences

Percent Similarity: 87.137 Percent Identity: 78.008
Protox-1.Pep x Mzprotox-1.Pep

```
 51 GGTTITTDCVIVGGGISGLCIAQALATKHPDAAPNLIVTEAKDRVGGNII 100
       ..|||:|||||||||.|||||||:|  :..:::||||:.|.|||||.
  1 ....NSADCVVVGGGISGLCTAQALATRH..GVGDVLVTEARARPGGNIT 44

101 T..REENGFLWEEGPNSFQPSDPMLTMVVDSGLKDDLVLGDPTAPRFVLW 148
    |  |.|:|:|||||||||||||||:|||.||||||||||:|||.|||||||
 45 TVERPEEGYLWEEGPNSFQPSDPVLTMAVDSGLKDDLVFGDPNAPRFVLW 94

149 NGKLRPVPSKLTDLPFFDLMSIGGKIRAGFGALGIRPSPPGREESVEEFV 198
    :|||||||||  .||||||||||.||:|||:|||||||.||||||||||||
 95 EGKLRPVPSKPADLPFFDLMSIPGKLRAGLGALGIRPPPPGREESVEEFV 144

199 RRNLGDEVFERLIEPFCSGVYAGDPSKLSMKAAFGKVWKLEQNGGSIIGG 248
    ||||||.||||||||||||||||||||||||||||:||:.|||||||||
145 RRNLGAEVFERLIEPFCSGVYAGDPSKLSMKAAFGKVWRLEETGGSIIGG 194

249 TFKAIQERKNAPKAERDPRLPKPQGQTVGSFRKGLRMLPEAISARLGSKV 298
    |:|.||||...||:.||:||||||.||||:|||||| |||:||...|||||
195 TIKTIQERSKNPKPPRDARLPKPKGQTVASFRKGLAMLPNAITSSLGSKV 244

299 KLSWKLSGITKLESGGYNLTYETPDGLVSVQSKSVVMTVPSHVASGLLRP 348
    ||||||.:||| :. || |.||||:|:||||.|||:||:||.|||.:|||
245 KLSWKLTSITKSDDKGYVLEYETPEGVVSVQAKSVIMTIPSYVASNILRP 294

349 LSESAANALSKLYYPPVAAVSISYPKEAIRTECLIDGELKGFGQLHPRTQ 398
    ||..||:|||::|||||||||.:||||||||.||||||||.||||||||.|
295 LSSDAADALSRFYYPPVAAVTVSYPKEAIRKECLIDGELQGFGQLHPRSQ 344

399 GVETLGTIYSSSLFPNRAPPGRILLLNYIGGSTNTGILSKSEGELVEAVD 448
    ||||||||||||||||||.||:||||||||.|||||:||.|:|||||||
345 GVETLGTIYSSSLFPNRAPDGRVLLLNYIGGATNTGIVSKTESELVEAVD 394

449 RDLRKMLIKPNSTDPLKLGVRVWPQAIPQFLVGHFDILDTAKSSLTSSGY 498
    ||||||||.....||| ||||||||||||||||:|:|:.||..|..:||
395 RDLRKMLINSTAVDPLVLGVRVWPQAIPQFLVGHLDLLEAAKAALDRGGY 444

499 EGLFLGGNYVAGVALGRCVEGAYETAIEVNNFMSRYAYK* 538
    :|||||||||||||||||||||||.| ::.:|:.:|||||
445 DGLFLGGNYVAGVALGRCVEGAYESASQISDFLTKYAYK* 484
```

Identical residues are denoted by the vertical bar between the two sequences. Alignment is performed using the GAP program described in Deveraux *et al., Nucleic Acids Res. 12*:387-395 (1984).

TABLE 2

Comparison of the Arabidopsis (SEQ ID No. 4) and Maize (SEQ ID NO. 8) Protox-2 Amino Acid Sequences

Percent Similarity: 75.889 Percent Identity: 57.905
Protox-2.Pep x Mzprotox-2.Pep

```
  1 ..........................MASGAVAD.HQIEAVSGKRVAV 21
                                .|  |:|: .:  |..::.|||
  1 MLALTASASSASSHPYRHASAHTRRPRLRAVLAMAGSDDPRAAPARSVAV 50

 22 VGAGVSGLAAAYKLKSRGLNVTVFEADGRVGGKLRSVMQNGLIWDEGANT 71
    |||||||||||||:|: .|:||||||||.:|.|||:|.  :.|::|||||||
 51 VGAGVSGLAAAYRLRQSGVNVTVFEAADRAGGKIRTNSEGGFVWDEGANT 100

 72 MTEAEPEVGSLLDDLGLREKQQFPISQKKRYIVRNGVPVMLPTNPIELVT 121
    |||:| |.:.|:|||||.:|||:| ||.|||||::|.|.::|.:||.|:.
101 MTEGEWEASRLIDDLGLQDKQQYPNSQHKRYIVKDGAPALIPSDPISLMK 150

122 SSVLSTQSKFQILLEPFLWKK....KSSKVSDASAEESVSEFFQRHFGQE 167
    ||||||.||:.:::||||:||    .|:|||:.  .|||:.| :||||.|
151 SSVLSTKSKIALFFEPFLYKKANTRNSGKVSEEHLSESVGSFCERHFGRE 200

168 VVDYLIDPFVGGTSAADPDSLSMKHSFPDLWNVEKSFGSIIVGAIRTKFA 217
    ||||:|||||:||||:||:|||::|.||.|||:|:.:||:|||||  .|:|
201 VVDYFVDPFVAGTSAGDPESLSIRHAFPALWNLERKYGSVIVGAILSKLA 250

218 AKGGKSRDTKSSPGTKKGSRGSFSFKGGMQILPDTLCKSLSHDEINLDSK 267
    |||:. :. ..|.|.:::..|.||||.|||| | :.| ..:::.|::.|:..
251 AKGDPVKTRHDSSGKRRNRRVSFSFHGGMQSLINALHNEVGDDNVKLGTE 300

268 VLSLS..YNSGSRQENWSLSCVSHNETQRQ...NPHYDAVIMTAPLCNVK 312
    ||||.  :::..  :.||:|. |.:..:::   |. :||||||||:|||:
301 VLSLACTFDGVPALGRWSISVDSKDSGDKDLASNQTFDAVIMTAPLSNVR 350

313 EMKVMKGGQPFQLNFLPEINYMPLSVLITTFTKEKVKRPLEGFGVLIPSK 362
     ||. |||.|. |:|||.:::|:|||:::|.|.|:.||:|||||||||| |
351 RMKFTKGGAPVVLDFLPKMDYLPLSLMVTAFKKDDVKKPLEGFGVLIPYK 400

363 E.QKHGFKTLGTLFSSMMFPDRSPSDVHLYTTFIGGSRNQELAKASTDEL 411
    | ||||:||||||||||||||.|.| .|||||:|||:|.:|| |.|. |
401 EQQKHGLKTLGTLFSSMMFPDRAPDDQYLYTTFVGGSHNRDLAGAPTSIL 450
```

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412 KQVVTSDLQRLLGVEGEPVSVNHYYWRKAFPLYDSSYDSVMEAIDKMEND 461
    ||:||||||.:|||||||:|. |.| || .|||||: .|.||:|||:|||.:
451 KQLVTSDLKKLLGVEGQPTFVKHVYWGNAFPLYGHDYSSVLEAIEKMEKN 500

462 LPGFFYAGNHRGGLSVGKSIASGCKAADLVISYLESCSNDKKPNDSL* 509
    |||||||||  ::||.||. ||||:|||||.|||||| ......:
501 LPGFFYAGNSKDGLAVGSVIASGSKAADLAISYLESHTKHNNSH*... 545
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EXAMPLE 4: Isolation of a contaminating yeast Protox clone from an Arabidopsis cDNA library

In an effort to identify any rare cDNAs with protox activity, a second screen of the pFL61 Arabidopsis library was done as before, again yielding hundreds of complementing clones. Approximately 600 of these were patched individually onto gridded plates and incubated at 28°C for 18 hours. Duplicate filter lifts were made onto Colony/Plaque screen (NEN) membranes according to the manufacturer's instructions. The Protox-1 and Protox-2 cDNAs were removed from their vectors by digestion with EcoRI/XhoI and by NotI, respectively. The inserts were separated by gel electrophoresis in 1.0% SeaPlaque GTG (FMC) agarose, excised, and ^{32}P-labeled by random priming (Life Technologies). One set of lifts was hybridized with each probe. Hybridization and wash conditions were as described in Church and Gilbert, 1984.

Colonies (~20) that failed to show clear hybridization to Protox-1 or Protox-2 were amplified in liquid culture and plasmid DNA was prepared. The DNA's were digested with NotI, duplicate samples were run on a 1.0% agarose gel, and then Southern blotted onto a Gene Screen Plus (NEN) filter [New England Nuclear]. Probes of the two known Protox genes were labeled and hybridized as before. There were two identical clones that were not Protox-1 or Protox-2. This clone was shown to recomplement the SASX38 mutant, although it grows very slowly, and was designated Protox-3.

Protox-3, in the pFL61 vector, was deposited June 8, 1994 as pWDC-5 (NRRL #B-21280). This coding sequence has been determined to be derived from yeast DNA which was present as a minor contaminant in the *Arabidopsis* cDNA library. The yeast DNA encoding protox-3 contained in pWDC-5 is set forth in SEQ ID NO:9 below.

EXAMPLE 5: Demonstration of plant protox clone sensitivity to protox inhibitory herbicides in a bacterial system.

Liquid cultures of Protox-1/SASX38, Protox-2/SASX38 and pBluescript/XL1-Blue were grown in L amp^{100}. One hundred microliter aliquots of each culture were plated on L amp^{100} media containing various concentrations (1.0nM-10mM) of a protox inhibitory aryluracil herbicide of formula XVII. Duplicate sets of plates were incubated for 18 hours at 37^{o} C in either low light or complete darkness.

The protox^{+} *E. coli* strain XL1-Blue showed no sensitivity to the herbicide at any concentration, consistent with reported resistance of the native bacterial enzyme to similar herbicides. The Protox-1/SASX38 was clearly sensitive, with the lawn of bacteria almost entirely eliminated by inhibitor concentrations as low as 10nM. The Protox-2/SASX38 was also sensitive, but only at a higher concentration (10 M) of the herbicide. The effect of the herbicide on both plant protox strains was most dramatic in low light, but was also apparent on plates maintained entirely in the dark. The toxicity of the herbicide was entirely eliminated by the addition of 20mg/ml hematin to the plates.

The different herbicide tolerance between the two plant Protox strains is likely the result of differential expression from these two plasmids, rather than any inherent difference in enzyme sensitivity. Protox-1/SASX38 grows much more slowly than Protox-2/SASX38 in any heme-deficient media. In addition, the MzProtox-2/SASX38 strain, with a growth rate comparable to Arab Protox-1/SASX38, is also very sensitive to herbicide at the lower (10-100nM) concentrations. Initial characterization of the yeast Protox-3 clone indicated that it also is herbicide sensitive.

EXAMPLE 6: Selecting for plant protox genes resistant to protox-inhibitory herbicides in the E. coli expression system.

Inhibition of plant protox enzymes in a bacterial system is useful for large-scale screening for herbicide-resistant mutations in the plant genes. Initial dose response experiments, done by plating from liquid cultures, gave rise to high frequency "resistant" colonies even at high concentrations of herbicide. This resistance was not plasmid-borne, based on retransformation/herbicide sensitivity assay. Transforming Protox

plasmids into the SASX38 mutant and plating directly onto plates containing herbicide reduces this background problem almost entirely.

The plant protox plasmids are mutagenized in a variety of ways, using published procedures for chemical (*e.g.* sodium bisulfite (Shortle *et al., Methods Enzymol. 100*:457-468 (1983); methoxylamine (Kadonaga *et al., Nucleic Acids Res. 13*:1733-1745 (1985); oligonucleotide-directed saturation mutagenesis (Hutchinson *et al., Proc. Natl. Acad. Sci. USA, 83*:710-714 (1986); or various polymerase misincorporation strategies (*see, e.g.* Shortle *et al., Proc. Natl. Acad. Sci. USA, 79*:1588-1592 (1982); Shiraishi *et al., Gene 64*:313-319 (1988); and Leung *et al., Technique 1*:11-15 (1989)). The expected up-promoter mutants from whole-plasmid mutagenesis are eliminated by recloning the coding sequence into a wild-type vector and retesting. Given that higher expression is likely to lead to better growth in the absence of herbicide, a visual screen for coding sequence mutants is also possible.

Any plant protox gene expressing herbicide resistance in the bacterial system may be engineered for optimal expression and transformed into plants using standard techniques as described herein. The resulting plants may then be treated with herbicide to confirm and quantitate the level of resistance conferred by the introduced protox gene.

EXAMPLE 7: Constructs for Expression of herbicide-resistant microbial protox gene(s) in plants.

The coding sequences for the *B. subtilis* protox gene *hemY* (Hansson and Hederstedt, *J. Bacteriol. 174*: 8081 (1992); Dailey *et al., J. Biol. Chem. 269*: 813 (1994)) and for the *E. coli* protox gene *hemG* (Sasarman *et al., Can. J. Microbiol. 39:* 1155 (1993)) were isolated from laboratory strains by PCR amplification using standard conditions and flanking primers designed from the published sequences. These genes are known to code for herbicide-resistant forms of the protox enzyme.

Using standard techniques of overlapping PCR fusion (Ausubel *et al.*, Current Protocols in Molecular Biology. John Wiley & Sons, Inc. (1994)), both bacterial genes were fused to two different *Arabidopsis* chloroplast transit peptide sequences (CTPs). The first was the CTP from the acetohydroxy acid synthase (AHAS, Mazur *et al., Plant*

The second was from the *Arabidopsis* plastocyanin gene (Vorst *et al., Gene 65*: 59 (1988)), which has a bipartite transit peptide. The amino terminal portion of this CTP targets the protein into the chloroplast, where the carboxy terminus routes it into the thylakoid membranes. All four gene fusions were cloned behind the 2X35S promoter in a binary expression vector designed for production of transgenic plants by agrobacterium transformation.

Following isolation of the *Arabidopsis* and maize protox cDNAs, the chloroplast transit peptide from Protox-1 or MzProtox-1 may also be fused to the two bacterial protox proteins in the same manner as above.

The vectors described above may then be transformed into the desired plant species and the resulting transformants assayed for increased resistance to herbicide.

EXAMPLE 8: Domain switching between Arabidopsis/B. subtilis genes to produce chimeric, herbicide resistant protox.

One approach that may be used to generate a protox gene which is both herbicide resistant and capable of providing effective protox enzymatic activity in a plant is to fuse portion(s) of a bacterial and plant protox gene. The resulting chimeric genes may then be screened for those which are capable of providing herbicide resistant protox activity in a plant cell. For instance, the *Arabidopsis* and the *B. subtilis* (*hemY*) protox peptide sequences are reasonably colinear with regions of high homology. The *hemY* coding sequence is cloned into pBluescript and tested for its ability to express herbicide-resistant protox activity in SASX38. Protox-1/*hemY* chimeric genes are constructed using fusion PCR techniques, followed by ligation back into the pBluescript vector. The initial exchange is approximately in the middle of the proteins. These fusions are tested for protox function by complementation, and then assayed for herbicide resistance by plating on herbicide with intact Protox-1 and *hemY* controls.

EXAMPLE 9: Production of herbicide-tolerant plants by overexpression of plant protox genes.

To express the *Arabidopsis* or maize protein in transgenic plants, the appropriate full length cDNA was inserted into the plant expression vector pCGN1761ENX, which was derived from pCGN1761 as follows. pCGN1761 was digested at its unique EcoRI site

and ligated to a double-stranded DNA fragment comprised of two oligonucleotides of sequence 5' AAT TAT GAC GTA ACG TAG GAA TTA GCG GCCC GCT CTC GAG T 3' (SEQ ID NO: 11) and 5' AAT TAC TCG AGA GCG GCC GCG AAT TCC TAC GTT ACG TCA T 3' (SEQ ID NO: 12). The resulting plasmid, pCGN1761ENX, contained unique EcoRI, NotI, and XhoI sites that lie between a duplicated 35S promoter from cauliflower mosaic virus (Kay *et al., Science 236:*1299-1302 (1987)) and the 3' untranslated sequences of the *tml* gene of *Agrobacterium tumefaciens*. This plasmid is digested and ligated to a fragment resulting from restriction enzyme digestion of one of the plasmids bearing a protox cDNA, such that it carries the complete protox cDNA. From this plasmid is excised an XbaI fragment comprising the *Arabidopsis* protox cDNA flanked by a duplicated 35S promoter and the 3' untranslated sequences of the *tml* gene of *A. tumefaciens*. This XbaI fragment is inserted into the binary vector pCIB200 at its unique XbaI site, which lies between T-DNA border sequences. The resulting plasmid, designated pCIB200protox, is transformed into *A. tumefaciens* strain CIB542. *See,e.g.* Uknes *et al., Plant Cell 5:*159-169 (1993).

Leaf disks of Nicotiana tabacum cv. Xanthi-nc are infected with A. tumefaciens CIB542 harboring pCIB200IGPD as described by Horsch *et al, Science 227:* 1229 (1985). Kanamycin-resistant shoots from 15 independent leaf disks are transferred to rooting medium, then transplanted to soil and the resulting plants grown to maturity in the greenhouse. Seed from these plants are collected and germinated on MS agar medium containing kanamycin. Multiple individual kanamycin resistant seedlings from each independent primary transformant are grown to maturity in the greenhouse, and their seed collected. These seeds are germinated on MS agar medium containing kanamycin.

Plant lines that give rise to exclusively kanamycin resistant seedlings are homozygous for the inserted gene and are subjected to further analysis. Leaf disks of each of the 15 independent transgenic lines are excised with a paper punch and placed onto MS agar containing various increasing concentrations of a protox inhibitory herbicide.

After three weeks, two sets of 10 disks from each line were weighed, and the results recorded. Transgenic lines more resistant to the inhibitor than wild type, non-transformed plants are selected for further analysis.

RNA is extracted from leaves of each of these lines. Total RNA from each independent homozygous line, and from non-transgenic control plants, is separated by agarose gel electrophoresis in the presence of formaldehyde (Ausubel *et al.*, Current Protocols in Molecular Biology, Wiley & Sons, New York (1987)). The gel is blotted to nylon membrane (Ausubel *et al.*, supra.) and hybridized with the radiolabeled *Arabidopsis* protox cDNA. Hybridization and washing conditions are as described by Church and Gilbert, *Proc. Natl. Acad. Sci. USA 81*:1991-1995 (1984). The filter is autoradiographed, and intense RNA bands corresponding to the protox transgene are detected in all herbicide-tolerant transgenic plant lines.

To further evaluate resistance of the protox-overexpressing line, plants are grown in the greenhouse and treated with various concentrations of a protox-inhibiting herbicide.

EXAMPLE 10: Growth of tobacco cell suspension cultures

Media:

MX1: This medium consists of Murashige and Skoog ("MS", T. Murashige *et al.*, *Physiol. Plant. 15*:473-497, 1962) major salts, minor salts and Fe-EDTA (Gibco # 500-1117; 4.3 g/l), 100 mg/1 myo-inositol, 1 mg/1 nicotinic acid, 1 mg/1 pyridoxine-HC1, 10 mg/1 thiamine -HC1, 2-3 g/1 sucrose, 0.4 mg/1 2,4-dichlorophenoxyacetic acid, and 0.04 mg/1 kinetin, pH 5.8. The medium is sterilized by autoclaving.

N6: This medium comprises macroelements, microelements and Fe-EDTA as described by C-C. Chu *et al.*, *Scientia Sinica 18*:659 (1975), and the following organic compounds: Pyridoxine-HC1 (0.5 mg/1), thiamine-HC1 (0.1 mg/1), nicotinic acid (0.5 mg/1), glycine (2.0 mg/1), and sucrose (30.0 g/1). The solution is autoclaved. The final pH is 5.6.

Remarks: Macroelements are made up as a 10 X concentrated stock solution, and microelements as a 1000 X concentrated stock solution. Vitamin stock solution is normally prepared 100 X concentrated.

Suspension cultured cells of *Nicotiana tabacum*, line S3 [Harms and DiMaio, J Plant Physiol 137, 513-519, 1991] are grown in liquid culture medium MX1. 100 ml Erlenmeyer flasks containing 25 ml medium MX1 are inoculated with 10 ml of a cell culture previously grown for 7 days. Cells are incubated at 25 C in the dark on an orbital shaker at 100 rpm (2 cm throw). Cells are subcultured at 7 day intervals by inoculating an aliquot sample into fresh medium, by decanting or pipetting off around 90% of the cell suspension followed by replenishing fresh medium to give the desired volume of suspension. 5-8 grams of fresh weight cell mass are produced within 10 days of growth from an inoculum of 250-350 mg cells.

EXAMPLE 11: Production of tobacco cell cultures tolerant to herbicidal protox inhibitors by plating cells on solidified selection medium

Cells are pregrown as in Example 10. Cells are harvested by allowing cells to sediment, or by brief centrifugation at 500 x g, and the spent culture medium is removed. Cells are then diluted with fresh culture medium to give a cell density suitable for cell plating, about 10,000 colony forming units per ml. For plating, cells in a small volume of medium (approx. 1 ml) are evenly spread on top of solidified culture medium (MX1, 0.8% agar) containing the desired concentration of the inhibitor. About 20-30 ml of medium are used per 10 cm Petri plate. The suitable inhibitor concentration is determined from a dose-response curve (Example 14), and is at least twofold higher than the IC_{50} of sensitive wild-type cells.

Culture plates containing cells spread onto selection medium are incubated under normal growth conditions at 25-28 C in the dark until cell colonies are formed. Emerging cell colonies are transferred to fresh medium containing the inhibitor in the desired concentration.

In a preferred modification of the described method the pregrown suspension of cultured cells is first spread in a small volume of liquid medium on top of the solidified medium. An equal amount of warm liquid agar medium (1.2-1.6% agar) kept molten at

around 40 C is added and the plate gently but immediately swirled to spread the cells evenly over the medium surface and to mix cells and agar medium, before the medium solidifies.

Alternatively, the cells are mixed with the molten agar medium prior to spreading on top of the selection medium. This method has the advantage that the cells are embedded and immobilized in a thin layer of solidified medium on top of the selection medium. It allows for better aeration of the cells as compared to embedding cells in the whole volume of 20-30 ml.

EXAMPLE 12: Production of tobacco cell cultures tolerant to a herbicidal protox inhibitor by growing cells in liquid selection medium

Cells cultured as in Example 10 are inoculated at a suitable cell density into liquid medium MX1 containing the desired concentration of a herbicidal protox inhibitor. Cells are incubated and grown as in Example 10. Cells are subcultured, as appropriate depending on the rate of growth, using fresh medium containing the desired inhibitor concentration after a period of 7-10 days.

Depending on the inhibitor concentration used, cell growth may be slower than in the absence of inhibitor.

EXAMPLE 13: Production of tobacco cells with enhanced levels of protox enzyme

In order to obtain cell cultures or callus with enhanced levels of protox enzyme, suspension cultures or callus are transferred, in a step-wise manner, to increasingly higher concentrations of herbicidal protox inhibitor. In particular, the following steps are performed:

Cell colonies emerging from plated cells of Example 11 are transferred to liquid MX1 medium containing the same concentration of protox inhibitor as used in the selection according to Example 11 in order to form suspension cultures. Alternatively, selected cell suspension cultures of Example 12 are subcultured in liquid MX1 medium containing the same concentration of protox inhibitor as used for selection according to Example 12.

Cultures are subcultured 1-20 times at weekly intervals and are then subcultured into MX1 medium containing the next higher herbicide concentration. The cells are cultured for 1-10 subcultures in medium containing this higher concentration of herbicide. The cells are then transferred to MX1 medium containing the next higher concentration of herbicide.

Alternatively, pieces of selected callus of Example 11 are transferred to solidified MX1 medium supplemented with the desired herbicide concentration. Transfer to higher herbicide concentrations follows the procedure outlined in the preceding paragraph except that solidified medium is used.

EXAMPLE 14: Measuring herbicide dose-dependent growth of cells in suspension cultures

In order to obtain a dose-response curve the growth of cells at different concentrations of herbicide is determined. Suspension culture cells of herbicidal protox inhibitor sensitive wild-type tobacco cells S3 and herbicide tolerant selected or transgenic cells S3 and herbicide tolerant selected or transgenic cells are pregrown in liquid medium as in Example 11 at a high cell density for 2-4 days. The cells are washed free of spent medium and fresh medium without herbicide is added to give the desired cell density (about 150 mg FW cells per ml of suspension). A sample of 2.5 ml of cell suspension, containing approx. 250-300 mg FW cells, is then inoculated into approx. 30 ml of liquid medium of desired herbicide concentration contained in a 100 ml Erlenmeyer flask. Care is taken to inoculate the same amount of cells into each flask. Each flask contains an equal volume of medium. 3-6 replicate flasks are inoculated per herbicide concentration. The herbicide concentration is selected from zero (= control), 0.1 ppb, 0.3 ppb, 1 ppb, 3 ppb, 10 ppb, 30 ppb, 100 ppb, 300 ppb, 1000 ppb, 3000 ppb, and 10,000 ppb. Several samples of inoculated cells are also taken at the time of inoculation to determine the mass of cells inoculated per flask.

Cells are then incubated for growth under controlled conditions at 28 in the dark for 10 days. The cells are harvested by pouring the contents of each flask onto a filter paper disk attached to a vacuum suction device to remove all liquid and to obtain a

mass of reasonably dry fresh cells. The fresh mass of cells is weighed. The dry weight of samples may be obtained after drying.

Cell growth is determined and expressed as cell gain within 10 days and expressed as a percentage relative to cells grown in the absence of herbicide according to the formula: (final mass of herbicide-grown cells minus inoculum mass x 100 divided by final mass of cells grown without herbicide minus inoculum mass). IC_{50} values are determined from graphs of plotted data (relative cell mass vs. herbicide concentration). IC_{50} denotes the herbicide concentration at which cell growth is 50% of control growth (cells grown in the absence of herbicide).

In a modification of the method several pieces of callus derived from a herbicide resistant cell culture, as obtained in Examples 11 and 13, are transferred to solidified callus culture medium containing the different herbicide concentrations. Relative growth is determined after a culture period of 2-6 weeks be weighing callus pieces and comparing to a control culture grown in medium without herbicide. However, the suspension method is preferred for its greater accuracy.

EXAMPLE 15: Determination of cross tolerance

In order to determine the extent at which cells show tolerance to analogous or other herbicides, Example 14 is repeated by growing cells in increasing concentrations of chosen herbicides. The relative growth of the cells and their IC_{50} value is determined for each herbicide for comparison.

EXAMPLE 16: Determining the stability of the herbicide tolerance phenotype over time

In order to determine whether the herbicide tolerant phenotype of a cell culture is maintained over time, cells are transferred from herbicide containing medium to medium without herbicide. Cells are grown, as described in Example 10, in the absence of herbicide for a period of 3 months, employing regular subculturing at suitable intervals (7-10 days for suspension cultures; 3-6 weeks for callus cultures). A known quantity of cells is then transferred back to herbicide containing medium and cultured for 10 days (suspension cultures) or 4 weeks (callus cultures). Relative growth is determined as in Example 14.

EXAMPLE 17: Induction and culture of embryogenic callus from corn scutellum tissue

Ears are harvested from self pollinated corn plants of the inbred line Funk 2717 12-14 days post pollination. Husks are removed and the ears are sterilized for about 15 minutes by shaking in a 20% solution of commercial Chlorox bleach with some drops of detergent added for better wetting. Ears are then rinsed several times with sterile water. All further steps are performed aseptically in a sterile air flow hood. Embryos of 1.5-2.5 mm length are removed from the kernels with a spatula and placed, embryo axis downwards, onto MS culture medium containing 2 mg/1 2,4-dichlorophenoxyacetic acid (2,4-D) and 3% sucrose, solidified with 0.24% Gelrite^{R}.

Embryogenic callus forms on the scutellum tissue of the embryos within 2-4 weeks of culture at about 28 C in the dark. The callus is removed from the explant and transferred to fresh solidified MS medium containing 2 mg/1 2,4-D. The subculture of embryogenic callus is repeated at weekly intervals. Only callus portions having an embryogenic morphology are subcultured.

EXAMPLE 18: Selection of corn cell cultures tolerant to herbicidal protox inhibitors

a) Selection using embryogenic callus: Embryogenic callus of Example 17 is transferred to callus maintenance medium consisting of N6 medium containing 2 mg/1 2,4-D, 3% sucrose and protox inhibitor at a concentration sufficient to retard growth, but that does not affect the embyrogenicity of the culture, and solidified with 0.24% Gelrite^{R}. To increase the frequency of herbicide tolerant mutations, cultures can be pretreated before selection with a chemical mutagen, *e.g.* ethylmethane sulfonate, or a physical mutagen, *e.g.* UV light, at a concentration just below the concentration at which growth inhibition is detected, as determined in Example 14. Cultures are

incubated at 28 C in the dark. After 14 days growing callus is transferred to fresh medium of the same composition. Only cultures with the desired embryogenic morphology known as friable embryogenic callus of type II morphology are subcultured. Cultures are propagated by subculturing at weekly intervals for two to ten subcultures on fresh medium whereby only the fastest growing cultures are subcultured. The fast growing callus is then transferred to callus maintenance medium containing a protox inhibiting herbicide at a suitable concentration as defined in Example 11. When callus grows well on this herbicide concentration, usually after about five to ten weekly subcultures, the callus is transferred to callus maintenance medium containing a three-fold higher concentration of inhibitor, and subcultured until a well growing culture is obtained. This process is repeated using medium containing protox inhibitor at a concentration 10-fold higher than the original suitable concentration, and again with medium containing 20-fold and 40-fold higher concentrations.

When sufficient callus has been produced it is transferred to regeneration medium suitable for embryo maturation and plant regeneration. Embryogenic callus growing on each of the herbicide concentrations used is transferred to regeneration medium.

b) Selection using embryogenic suspension cultures: Embryogenic suspension cultures of corn Funk inbred line 2717 are established according to Example 24 and maintained by subculturing at weekly intervals to fresh liquid N6 medium containing 2 mg/1 2,4-D. To increase the frequency of herbicide tolerant mutations, cultures can be treated at this time with a chemical mutagen, *e.g.* ethylmethane sulfonate, at a concentration just below the concentration at which growth inhibition is detected, as determined in Example 14. For selection, the cultures are transferred to liquid N6 medium containing 2 mg/1 2,4-D and a concentration of inhibitor sufficient to retard growth, but that does not affect the embyrogenicity of the culture. Cultures are grown on a shaker at 120 rpm at 28 C in the dark. At weekly intervals, the medium is removed and fresh medium added. The cultures are diluted with culture medium in accord with their growth to maintain about 10 ml of packed cell volume per 50 ml of medium. At each subculture, cultures are inspected and only fast growing cultures with

two to ten subcultures in N6 medium containing, cultures are increasing in growth rate at least two- to threefold per weekly subculture. The cultures are then transferred to N6 medium containing 2 mg/l 2,4-D and a three-fold higher dose of inhibitor than originally used. Growing cultures are repeatedly subcultured in this medium for another two to ten subcultures as described above. Fast growing cultures with the desired friable embryogenic morphology are selected for further subculture. Fast growing cultures are then transferred to N6 medium containing 2 mg 2,4-D and a ten-fold higher concentration of inhibitor than originally used, and the process of subculturing growing cultures with the desired friable embryogenic morphology is repeated for two to ten subcultures until fast growing cultures are obtained. These cultures are then transferred to N6 medium containing 2 mg/1 2,4-D and a 30-fold higher concentration of inhibitor than originally used.

For regeneration of plants from each embryogenic suspension culture selected with the mentioned herbicide concentration level, the cultures are first transferred onto N6 medium solidified with 0.24% Gelrite^{R} and containing 2 mg/1 2,4-D and, optionally, the concentration of inhibitor in which the cultures have been growing, to produce embryogenic callus. The embryogenic callus is subcultured onto fresh callus maintenance medium until a sufficient amount of callus is obtained for regeneration. Only cultures with the desired embryogenic morphology are subcultured.

EXAMPLE 19: Regeneration of corn plants form selected callus or suspension culture

Plants are regenerated from the selected embryogenic callus cultures of Example 13 by transferring to fresh regeneration medium. Regeneration media used are: 0N6 medium consisting of N6 medium lacking 2,4-3, or N61 consisting of N6 medium containing 0.25 mg/1 2,4-D and 10 mg/1 kinetin (6-furfurylaminopurine), or N62 consisting of N6 medium containing 0.1 mg/1 2,4-D and 1 mg/1 kinetin, all solidified with 0.24% Gelrite^{R}. Cultures are grown at 28 C in the light (16 h per day of 10-100 μEinsteins/m^{2}sec from white fluorescent lamps). The cultures are subcultured every two weeks onto fresh medium. Plantlets develop within 3 to 8 weeks. Plantlets at least 2 cm tall are removed from adhering callus and transferred to root promoting medium.

Different root promoting media are used. The media consist of N6 or MS medium lacking vitamins with either the usual amount of salts or with salts reduced to one half, sucrose reduced to 1 g/1, and further either lacking growth regulating compounds or containing 0.1 mg/1 a-naphthaleneacetic acid. Once roots are sufficiently developed, plantlets are transplanted to a potting mixture consisting of vermiculite, peat moss and garden soil. At transplanting all remaining callus is trimmed away, all agar is rinsed off and the leaves are clipped about half. Plantlets are grown in the greenhouse initially covered for some days with an inverted clear plastic cup to retain humidity and grown with shading. After acclimatization plants are repotted and grown to maturity. Fertilizer Peters 20-20-20 [Grace Sierra] is used to ensure healthy plant development. Upon flowering plants are pollinated, preferably self pollinated.

EXAMPLE 20: Construction of Plant Transformation Vectors

Numerous transformation vectors are available for plant transformation, and the genes of this invention can be used in conjunction with any such vectors. The selection of vector for use will depend upon the preferred transformation technique and the target species for transformation. For certain target species, different antibiotic or herbicide selection markers may be preferred. Selection markers used routinely in transformation include the *nptII* gene which confers resistance to kanamycin and related antibiotics (Messing & Vierra, *Gene 19:* 259-268 (1982); Bevan *et al.*, *Nature 304:*184-187 (1983)), the *bar* gene which confers resistance to the herbicide phosphinothricin (White *et al.*, *Nucl Acids Res 18:* 1062 (1990), Spencer *et al.* Theor Appl Genet 79: 625-631(1990)), the *hph* gene which confers resistance to the antibiotic hygromycin (Blochinger & Diggelmann, *Mol Cell Biol 4:* 2929-2931), and the *dhfr* gene, which confers resistance to methotrexate (Bourouis *et al.*, *EMBO J. 2(7):* 1099-1104 (1983)).

(1) Construction of Vectors Suitable for *Agrobacterium* Transformation

Many vectors are available for transformation using *Agrobacterium tumefaciens.* These typically carry at least one T-DNA border sequence and include vectors such as pBIN19

(Bevan, *Nucl. Acids Res.* (1984)). Below the construction of two typical vectors is described.

Construction of pCIB200 and pCIB2001

The binary vectors pCIB200 and pCIB2001 are used for the construction of recombinant vectors for use with *Agrobacterium* and was constructed in the following manner. pTJS75kan was created by *NarI* digestion of pTJS75 (Schmidhauser & Helinski, *J Bacteriol. 164*: 446-455 (1985)) allowing excision of the tetracycline-resistance gene, followed by insertion of an *AccI* fragment from pUC4K carrying an NPTII (Messing & Vierra, *Gene 19*: 259-268 (1982); Bevan *et al.*, *Nature 304*: 184-187 (1983); McBride *et al.*, *Plant Molecular Biology 14*: 266-276 (1990)). *XhoI* linkers were ligated to the *EcoRV* fragment of pCIB7 which contains the left and right T-DNA borders, a plant selectable *nos/nptII* chimeric gene and the pUC polylinker (Rothstein *et al.*, *Gene 53:* 153-161 (1987)), and the *XhoI*-digested fragment was cloned into *SalI*-digested pTJS75kan to create pCIB200 (see also EP 0 332 104, example 19 [1338]). pCIB200 contains the following unique polylinker restriction sites: *EcoRI, SstI, KpnI, BglII, XbaI,* and *SalI. pCIB2001* is a derivative of pCIB200 which created by the insertion into the polylinker of additional restriction sites. Unique restriction sites in the polylinker of pCIB2001 are *EcoRI, SstI, KpnI, BglII, XbaI, SalI, MluI, BclI, AvrII, ApaI, HpaI,* and *StuI.* pCIB2001, in addition to containing these unique restriction sites also has plant and bacterial kanamycin selection, left and right T-DNA borders for *Agrobacterium*-mediated transformation, the RK2-derived *trfA* function for mobilization between *E. coli* and other hosts, and the *OriT* and *OriV* functions also from RK2. The pCIB2001 polylinker is suitable for the cloning of plant expression cassettes containing their own regulatory signals.

Construction of pCIB10 and Hygromycin Selection Derivatives thereof

The binary vector pCIB10 contains a gene encoding kanamycin resistance for selection in plants, T-DNA right and left border sequences and incorporates sequences from the wide host-range plasmid pRK252 allowing it to replicate in both *E. coli* and *Agrobacterium.* Its construction is described by Rothstein *et al.*, *Gene 53*: 153-161

(1987). Various derivatives of pCIB10 have been constructed which incorporate the gene for hygromycin B phosphotransferase described by Gritz *et al.*, *Gene 25:* 179-188 (1983)). These derivatives enable selection of transgenic plant cells on hygromycin only (pCIB743), or hygromycin and kanamycin (pCIB715, pCIB717) [Rothstein *et al.*, *Gene 53*: 153-161 (1987)].

(2) Construction of Vectors Suitable for non-*Agrobacterium* Transformation.

Transformation without the use of *Agrobacterium tumefaciens* circumvents the requirement for T-DNA sequences in the chosen transformation vector and consequently vectors lacking these sequences can be utilized in addition to vectors such as the ones described above which contain T-DNA sequences. Transformation techniques which do not rely on *Agrobacterium* include transformation via particle bombardment, protoplast uptake (*e.g.* PEG and electroporation) and microinjection. The choice of vector depends largely on the preferred selection for the species being transformed. Below, the construction of some typical vectors is described.

Construction of pCIB3064

pCIB3064 is a pUC-derived vector suitable for direct gene transfer techniques in combination with selection by the herbicide basta (or phosphinothricin). The plasmid pCIB246 comprises the CaMV 35S promoter in operational fusion to the *E. coli* GUS gene and the CaMV 35S transcriptional terminator and is described in the PCT published application WO 93/07278. The 35S promoter of this vector contains two ATG sequences 5' of the start site. These sites were mutated using standard PCR techniques in such a way as to remove the ATGs and generate the restriction sites *Sspl* and *PvuII*. The new restriction sites were 96 and 37 bp away from the unique *SalI* site and 101 and 42 bp away from the actual start site. The resultant derivative of pCIB246 was designated pCIB3025. The GUS gene was then excised from pCIB3025 by digestion with *SalI* and *SacI*, the termini rendered blunt and religated to generate plasmid pCIB3060. The plasmid pJIT82 was obtained from the John Innes Centre, Norwich and the a 400 bp *SmaI* fragment containing the *bar* gene from *Streptomyces viridochromogenes* was excised and inserted into the *HpaI* site of pCIB3060

(Thompson *et al.* EMBO J 6: 2519-2523 (1987)). This generated pCIB3064 which comprises the *bar* gene under the control of the CaMV 35S promoter and terminator for herbicide selection, a gene fro ampicillin resistance (for selection in *E. coli*) and a polylinker with the unique sites *SphI, PstI, HindIII,* and *BamHI.* This vector is suitable for the cloning of plant expression cassettes containing their own regulatory signals.

Construction of pSOG19 and pSOG35

pSOG35 is a transformation vector which utilizes the *E. coli* gene dihydrofolate reductase (DHFR) as a selectable marker conferring resistance to methotrexate. PCR was used to amplify the 35S promoter (~800 bp), intron 6 from the maize Adh1 gene (~550 bp) [Lou et al, Plant J 3: 393-403, 1993; Dennis et al, Nucl Acids Res 12: 3983-4000, 1984] and 18 bp of the GUS untranslated leader sequence from pSOG10. A 250 bp fragment encoding the *E. coli* dihydrofolate reductase type II gene was also amplified by PCR and these two PCR fragments were assembled with a *SacI-PstI* fragment from pBI221 (Clontech) which comprised the pUC19 vector backbone and the nopaline synthase terminator. Assembly of these fragments generated pSOG19 which contains the 35S promoter in fusion with the intron 6 sequence, the GUS leader, the DHFR gene and the nopaline synthase terminator. Replacement of the GUS leader in pSOG19 with the leader sequence from Maize Chlorotic Mottle Virus (MCMV) generated the vector pSOG35. pSOG19 and pSOG35 carry the pUC gene for ampicillin resistance and have *HindIII, SphI, PstI* and *EcoRI* sites available for the cloning of foreign sequences.

pSOG 10

This β-Glucuronidase (GUS) expression vector was derived from plasmid pBI121, purchased from Clonetech Laboratories, Palo Alto, California. Intron 6 of the maize Adh1 gene was amplified by PCR from plasmid pB428, described in Bennetzen et al., Proc. Natl. Acad. Sci, USA 81:4125-4128 (1987), using oligonucleotide primers SON0003 and SON0004.

SON0003: 5'-CTCGGATCCAGCAGATTCGAAGAAGGTACAG-3'

SON0004: 5'-ACGGGATCCAACTTCCTAGCTGAAAAATGGG-3'

The PCR reaction product was digested with restriction endonuclease BamHI, cleaving the BamHI site added on the 5' end of each PCR primer. The resulting DNA fragment was purified on an agarose gel and ligated into the BamHI site of pBI121, which is between the CaMV35S promoter and the GUS gene. The ligated DNA was transformed into *E.coli* and clones with the Adh1 intron 6 in the same orientation as the GUS gene were identified by restriction digest.

pSOG 19

This dihydrofolate reductase (DHFR) expression vector was derived by fusing the 35S promotor and Adh1 intron 6 of pSOG10 to the DHFR gene from plasmid pHCO, described in Bourouis and Jarry, EMBO J. 2: 1099-1104 (1983) The 35S promoter and Adh1 intron 6 were produced by PCR amplification of the fragment from pSOG10 using primers SON0031 and SON0010.

SON0031: 5'-CATGAGGGACTGACCACCCGGGGATC-3'
SON0010: 5'-AGCGGATAACAATTTCACACAGGA-3'

The resulting fragment was digested with restriction endonucleases PstI and BspHI and purified on an agarose gel.

The DHFR coding region was produced by PCR amplification of pHCO using primers SON0016 and SON0017.

SON0016: 5'-GCTACCATGGCCACATAGAACACC-3'
SON0017: 5'-CGAGAGCTCGCACTTCAACCTTG-3'

The resulting fragment was digested with restriction endonucleases NsoI and SacI and purified on an agarose gel.

The two fragments described above were ligated into a vector fragment prepared from pBI121 by digestion with restriction endonucleases PstI and SacI and purification

on an agarose gel. This three way ligation fused the 35S promoter-Adhl intron 6-DHFR gene-Nos terminator in correct order and orientation for functional expression in plants.

pSOG 30

This GUS expression vector was derived from pSOG 10 by the insertion of the maize chlorotic mottle virus (MCMV) leader, described in Lommel et al., Virology 181: 382-385 (1991), into the 35S-GUS gene non-translated leader by a three way ligation.

Both strands of the 17 bp MCMV capsid protein leader sequence plus appropriate restriction endonuclease sights were synthesized and annealed. The resulting double stranded fragment was degested with BamHI and NcoI and purified on an acrylamide gel.

The GUS gene coding region was amplified by PCR using primers SON0039 and SON0041 and pBI121 as a template.

SON0039: 5'-CGACATGGTACGTCCTGTAGAAACCCACA-3'
SON0041: 5'-ATCGCAAGACCGGCAACAGGATTC-3'

These primers added an NcoI site to the 5' end of GUS and a SacI site to the 3' end of GUS. The resulting fragment was digested with restriction endonucleases NcoI and SacI and purified on an agarose gel.

The GUS gene was removed from the plasmid pSOG 10 by digestion with restriction endonuclease SacI and partial digestion with restriction endonuclease BamHI. The resulting vector, which has a BamHI site and a SacI site in which to reinsert a coding region behind the 35S promoter-Adh1 intron 6, was purified on an agarose gel.

The three fragments described above were ligated in a three way ligation to produce a gene fusion with the structure: 35Spromoter-Adh1 intron 6-MCMV leader-GUS-Nos terminator, all in the pUC19 vector backbone.

pSOG 35

The DHFR selectable marker vector is identical to pSOG19, except that the MCMV leader is inserted in the non-translated leader of the DHFR gene to enhance translation. It was created in two steps. First the GUS coding region in pSOG32, a vector identical to pSOG30 except that it contains a modified Adh promoter rather than 35S, was replaced with DHFR coding region from pSOG19 by excising the GUS with NcoI and SacI and ligating in the DHFR as an NcoI-SacI fragment. This resulting in vector pSOG33 which has the gene structure Adh promoter-Adh1 intron 6-MCMV leader-DHFR coding region-Nos terminator, with a BglII site between the promoter and Intron and a SacI site between the coding region and the terminator. The BglII-SacI fragment was isolated by restriction endonuclease digestion and agarose gel purification, and ligated into the BamHI and SacI sites of pSOG30, replacing the Adh1 intronZ6-MCMV leader-GUS coding region of pSOG30 with the Adh1 intron 6-MCMV leader-DHFR coding region of pSOG33.

EXAMPLE 21: Construction of Plant Expression Cassettes

Gene sequences intended for expression in transgenic plants are firstly assembled in expression cassettes behind a suitable promoter and upstream of a suitable transcription terminator. These expression cassettes can then be easily transferred to the plant transformation vectors described above in Example 19.

Promoter Selection

The selection of a promoter used in expression cassettes will determine the spatial and temporal expression pattern of the transgene in the transgenic plant. Selected promoters will express transgenes in specific cell types (such as leaf epidermal cells, mesophyll cells, root cortex cells) or in specific tissues or organs (roots, leaves or flowers, for example) and this selection will reflect the desired location of expression of the transgene. Alternatively, the selected promoter may drive expression of the gene under a light-induced or other temporally regulated promoter. A further alternative is that the selected promoter be chemically regulated. This would provide the possibility of inducing expression of the transgene only when desired and caused by treatment with a chemical inducer.

Transcriptional Terminators

A variety of transcriptional terminators are available for use in expression cassettes. These are responsible for the termination of transcription beyond the transgene and its correct polyadenylation. Appropriate transcriptional terminators and those which are known to function in plants and include the CaMV 35S terminator, the *tml* terminator, the nopaline synthase terminator, the pea *rbcS* E9 terminator. These can be used in both monocotyledons and dicotyledons.

Sequences for the Enhancement or Regulation of Expression

Numerous sequences have been found to enhance gene expression from within the transcriptional unit and these sequences can be used in conjunction with the genes of this invention to increase their expression in transgenic plants.

Various intron sequences have been shown to enhance expression, particularly in monocotyledonous cells. For example, the introns of the maize *Adh1* gene have been found to significantly enhance the expression of the wild-type gene under its cognate promoter when introduced into maize cells. Intron 1 was found to be particularly effective and enhanced expression in fusion constructs with the chloramphenicol acetyltransferase gene (Callis *et al.*, Genes Develop. 1: 1183-1200 (1987)). In the same experimental system, the intron from the maize *bronze1* gene had a similar effect in enhancing expression (Callis *et al., supra*). Intron sequences have been routinely incorporated into plant transformation vectors, typically within the non-translated leader.

A number of non-translated leader sequences derived from viruses are also known to enhance expression, and these are particularly effective in dicotyledonous cells. Specifically, leader sequences from Tobacco Mosaic Virus (TMV, the "W-sequence"), Maize Chlorotic Mottle Virus (MCMV), and Alfalfa Mosaic Virus (AMV) have been shown to be effective in enhancing expression (*e.g.* Gallie *et al. Nucl. Acids Res. 15*: 8693-8711 (1987); Skuzeski *et al.* Plant Molec. Biol. 15: 65-79 (1990))

Targeting of the Gene Product Within the Cell

Various mechanisms for targeting gene products are known to exist in plants and the sequences controlling the functioning of these mechanisms have been characterized in some detail. For example, the targeting of gene products to the chloroplast is controlled by a signal sequence found at the amino terminal end of various proteins and which is cleaved during chloroplast import yielding the mature protein (*e.g.* Comai *et al.* J. *Biol. Chem. 263*: 15104-15109 (1988)). These signal sequences can be fused to heterologous gene products to effect the import of heterologous products into the chloroplast (van den Broeck *et al. Nature 313*: 358-363 (1985)). DNA encoding for appropriate signal sequences can be isolated from the 5' end of the cDNAs encoding the RUBISCO protein, the CAB protein, the EPSP synthase enzyme, the GS2 protein and many other proteins which are known to be chloroplast localized.

Other gene products are localized to other organelles such as the mitochondrion and the peroxisome (*e.g.* Unger *et al. Plant Molec. Biol. 13*: 411-418 (1989)). The cDNAs encoding these products can also be manipulated to effect the targeting of heterologous gene products to these organelles. Examples of such sequences are the nuclear-encoded ATPases and specific aspartate amino transferase isoforms for mitochondria. Targeting to cellular protein bodies has been described by Rogers *et al., Proc. Natl. Acad. Sci. USA 82*: 6512-6516 (1985)).

In addition sequences have been characterized which cause the targeting of gene products to other cell compartments. Amino terminal sequences are responsible for targeting to the ER, the apoplast, and extracellular secretion from aleurone cells (Koehler & Ho, *Plant Cell 2:* 769-783 (1990)). Additionally, amino terminal sequences in conjunction with carboxy terminal sequences are responsible for vacuolar targeting of gene products (Shinshi *et al., Plant Molec. Biol. 14*: 357-368 (1990)).

By the fusion of the appropriate targeting sequences described above to transgene sequences of interest it is possible to direct the transgene product to any organelle or cell compartment. For chloroplast targeting, for example, the chloroplast signal sequence from the RUBISCO gene, the CAB gene, the EPSP synthase gene, or the GS2 gene is fused in frame to the amino terminal ATG of the transgene. The signal sequence selected should include the known cleavage site and the fusion constructed

should take into account any amino acids after the cleavage site which are required for cleavage. In some cases this requirement may be fulfilled by the addition of a small number of amino acids between the cleavage site and the transgene ATG or alternatively replacement of some amino acids within the transgene sequence. Fusions constructed for chloroplast import can be tested for efficacy of chloroplast uptake by *in vitro* translation of *in vitro* transcribed constructions followed by *in vitro* chloroplast uptake using techniques described by (Bartlett *et al.* In: Edelmann *et al.* (Eds.) Methods in Chloroplast Molecular Biology, Elsevier. pp 1081-1091 (1982); Wasmann *et al. Mol. Gen. Genet. 205*: 446-453 (1986)). These construction techniques are well known in the art and are equally applicable to mitochondria and peroxisomes. The choice of targeting which may be required for expression of the transgenes will depend on the cellular localization of the precursor required as the starting point for a given pathway. This will usually be cytosolic or chloroplastic, although it may is some cases be mitochondrial or peroxisomal. The products of transgene expression will not normally require targeting to the ER, the apoplast or the vacuole.

The above described mechanisms for cellular targeting can be utilized not only in conjunction with their cognate promoters, but also in conjunction with heterologous promoters so as to effect a specific cell targeting goal under the transcriptional regulation of a promoter which has an expression pattern different to that of the promoter from which the targeting signal derives.

EXAMPLE 22: Transformation of Dicotyledons

Transformation techniques for dicotyledons are well known in the art and include *Agrobacterium*-based techniques and techniques which do not require *Agrobacterium*. Non-*Agrobacterium* techniques involve the uptake of exogenous genetic material directly by protoplasts or cells. This can be accomplished by PEG or electroporation mediated uptake, particle bombardment-mediated delivery, or microinjection. Examples of these techniques are described by Paszkowski *et al.*, *EMBO J 3:* 2717-2722 (1984), Potrykus *et al., Mol. Gen. Genet. 199:* 169-177 (1985), Reich *et al., Biotechnology 4:* 1001-1004 (1986), and Klein *et al., Nature 327*: 70-73 (1987). In each case the

transformed cells are regenerated to whole plants using standard techniques known in the art.

Agrobacterium-mediated transformation is a preferred technique for transformation of dicotyledons because of its high efficiency of transformation and its broad utility with many different species. The many crop species which are routinely transformable by *Agrobacterium* include tobacco, tomato, sunflower, cotton, oilseed rape, potato, soybean, alfalfa and poplar (EP 0 317 511 (cotton), EP 0 249 432 (tomato, to Calgene), WO 87/07299 (*Brassica*, to Calgene), US 4,795,855 (poplar)). *Agrobacterium* transformation typically involves the transfer of the binary vector carrying the foreign DNA of interest (*e.g.* pCIB200 or pCIB2001) to an appropriate *Agrobacterium* strain which may depend of the complement of *vir* genes carried by the host *Agrobacterium* strain either on a co-resident Ti plasmid or chromosomally (*e.g.* strain CIB542 for pCIB200 and pCIB2001 (Uknes *et al. Plant Cell 5*: 159-169 (1993)). The transfer of the recombinant binary vector to *Agrobacterium* is accomplished by a triparental mating procedure using *E. coli* carrying the recombinant binary vector, a helper *E. coli* strain which carries a plasmid such as pRK2013 and which is able to mobilize the recombinant binary vector to the target *Agrobacterium* strain. Alternatively, the recombinant binary vector can be transferred to *Agrobacterium* by DNA transformation (Höfgen & Willmitzer, Nucl. Acids Res. 16: 9877(1988)).

Transformation of the target plant species by recombinant *Agrobacterium* usually involves co-cultivation of the *Agrobacterium* with explants from the plant and follows protocols well known in the art. Transformed tissue is regenerated on selectable medium carrying the antibiotic or herbicide resistance marker present between the binary plasmid T-DNA borders.

EXAMPLE 23: Transformation of Monocotyledons

Transformation of most monocotyledon species has now also become routine. Preferred techniques include direct gene transfer into protoplasts using PEG or electroporation techniques, and particle bombardment into callus tissue. Transformations can be undertaken with a single DNA species or multiple DNA species (*i.e.* co-transformation) and both these techniques are suitable for use with this

invention. Co-transformation may have the advantage of avoiding complex vector construction and of generating transgenic plants with unlinked loci for the gene of interest and the selectable marker, enabling the removal of the selectable marker in subsequent generations, should this be regarded desirable. However, a disadvantage of the use of co-transformation is the less than 100% frequency with which separate DNA species are integrated into the genome (Schocher *et al. Biotechnology 4:* 1093-1096 (1986)).

Patent Applications EP 0 292 435 (to Ciba-Geigy), EP 0 392 225 (to Ciba-Geigy) and WO 93/07278 (to Ciba-Geigy) describe techniques for the preparation of callus and protoplasts from an élite inbred line of maize, transformation of protoplasts using PEG or electroporation, and the regeneration of maize plants from transformed protoplasts. Gordon-Kamm *et al., Plant Cell 2:* 603-618 (1990)) and Fromm *et al., Biotechnology 8:* 833-839 (1990)) have published techniques for transformation of A188-derived maize line using particle bombardment. Furthermore, application WO 93/07278 (to Ciba-Geigy) and Koziel *et al., Biotechnology 11:* 194-200 (1993)) describe techniques for the transformation of élite inbred lines of maize by particle bombardment. This technique utilizes immature maize embryos of 1.5-2.5 mm length excised from a maize ear 14-15 days after pollination and a PDS-1000He Biolistics device for bombardment.

Transformation of rice can also be undertaken by direct gene transfer techniques utilizing protoplasts or particle bombardment. Protoplast-mediated transformation has been described for *Japonica*-types and *Indica*-types (Zhang *et al., Plant Cell Rep 7*: 379-384 (1988); Shimamoto *et al. Nature 338*: 274-277 (1989); Datta *et al. Biotechnology 8:* 736-740 (1990)). Both types are also routinely transformable using particle bombardment (Christou *et al. Biotechnology 9:* 957-962 (1991)).

Patent Application EP 0 332 581 (to Ciba-Geigy) describes techniques for the generation, transformation and regeneration of Pooideae protoplasts. These techniques allow the transformation of *Dactylis* and wheat. Furthermore, wheat transformation was been described by Vasil *et al., Biotechnology 10:* 667-674 (1992)) using particle bombardment into cells of type C long-term regenerable callus, and also by Vasil *et al., Biotechnology 11:* 1553-1558 (1993)) and Weeks *et al., Plant Physiol.*

102: 1077-1084 (1993) using particle bombardment of immature embryos and immature embryo-derived callus. A preferred technique for wheat transformation, however, involves the transformation of wheat by particle bombardment of immature embryos and includes either a high sucrose or a high maltose step prior to gene delivery. Prior to bombardment, any number of embryos (0.75-1 mm in length) are plated onto MS medium with 3% sucrose (Murashige & Skoog, *Physiologia Plantarum 15*: 473-497 (1962)) and 3 mg/l 2,4-D for induction of somatic embryos which is allowed to proceed in the dark. On the chosen day of bombardment, embryos are removed from the induction medium and placed onto the osmoticum (*i.e.* induction medium with sucrose or maltose added at the desired concentration, typically 15%). The embryos are allowed to plasmolyze for 2-3 h and are then bombarded. Twenty embryos per target plate is typical, although not critical. An appropriate gene-carrying plasmid (such as pCIB3064 or pSG35) is precipitated onto micrometer size gold particles using standard procedures. Each plate of embryos is shot with the DuPont Biolistics® helium device using a burst pressure of ~1000 psi using a standard 80 mesh screen. After bombardment, the embryos are placed back into the dark to recover for about 24 h (still on osmoticum). After 24 hrs, the embryos are removed from the osmoticum and placed back onto induction medium where they stay for about a month before regeneration. Approximately one month later the embryo explants with developing embryogenic callus are transferred to regeneration medium (MS + 1 mg/liter NAA, 5 mg/liter GA), further containing the appropriate selection agent (10 mg/l basta in the case of pCIB3064 and 2 mg/l methotrexate in the case of pSOG35). After approximately one month, developed shoots are transferred to larger sterile containers known as "GA7s" which contained half-strength MS, 2% sucrose, and the same concentration of selection agent. Patent application 08/147,161 describes methods for wheat transformation and is hereby incorporated by reference.

EXAMPLE 24:Selecting for plant protox genes resistant to protox-inhibitory herbicides in the *E. coli* expression system

The plasmid pWDC-4, encoding the maize chloroplastic protox enzyme, is transformed into the random mutagenesis strain XL1-Red (Stratagene, La Jolla, CA).

The transformation is plated on L media containing 50 g/ml ampicillin and incubated for 48 hours at 37 C. Lawns of transformed cells are scraped from the plates and plasmid DNA prepared using the Wizard Megaprep kit (Promega, Madison, WI). Plasmid DNA isolated from this mutator strain is predicted to contain approximately one random base change per 2000 nucleotides (*see* Greener *et al., Strategies 7(2)*:32-34 (1994)).

The mutated plasmid DNA is transformed into the *hemG* mutant SASX38 (Sasarman *et al., J. Gen. Microbiol. 113:* 297 (1979) and plated on L media containing 100 g/ml ampicillin and on the same media containing various concentrations of protox-inhibiting herbicide. The plates are incubated for 2-3 days at 37 C. Plasmid DNA is isolated from all colonies that grow in the presence of herbicide concentrations that effectively kill the wild type strain. The isolated DNA is then transformed into SASX38 and plated again on herbicide to ensure that the resistance is plasmid-borne.

Mutated pWDC-4 plasmid DNA is again isolated from resistant colonies and the protox coding sequence is excised by digestion with EcoRI and XhoI. The excised protox coding sequence is then recloned into an unmutagenized pBluescript vector and retested for resistance to protox-inhibiting herbicide in the same manner described above.

This process eliminates non-coding sequence mutations which confer resistance such as up-promoter mutants (i.e. mutants whose resistance is due to mutations causing increased expression of unmodified protox) and leaves only mutants whose resistance is due to mutations in the protox coding sequence. The DNA sequence for all putative herbicide-tolerant protox genes identified through this process is determined and mutations are identified by comparison with the wild-type pWDC-4 protox sequence.

Using the procedure described above, a resistance mutation converting a C to a T at nucleotide 498 in the pWDC-4 sequence (SEQ ID No. 5) has been identified. The plasmid carrying this mutation has been designated pMzC-1Val. This change converts a GCT codon for alanine at amino acid 166 (SEQ ID No. 6) to a GTT codon for valine and results in a protox enzyme that is at least 10X more resistant to protox-inhibiting herbicide in the bacterial assay.

pMzC-1Val, in the pBluescript SK vector, was deposited on September 30, 1994 under the designation pWDC-8 with the Agricultural Research Culture Collection and given the deposit designation NRRL #21340.

The same strategy was used to screen for herbicide-resistant forms of the *Arabidopsis* Protox-1 gene in various vectors. One resistance mutation identified is a C to T change at nucleotide 689 in the pWDC-2 sequence (SEQ ID No. 1); this plasmid is designated pAraC-1Val. This change is identical to the pMzC-1Val mutant above, converting a GCT codon for alanine at amino acid 220 (SEQ ID No. 2) to a GTT codon for valine at the corresponding position in the *Arabidopsis* protox protein sequence.

A second resistant gene contains an A to G change at nucleotide 1307 in the pWDC-2 sequence (SEQ ID No. 1); this plasmid is designated pAraC-2Cys. This change converts TAC codon for tyrosine at amino acid 426 (SEQ ID No. 2) to a TGC codon for cysteine. The corresponding tyrosine codon in the maize protox-1 sequence at nucleotide position 1115-1117 (SEQ ID NO. 5; amino acid position 372 of SEQ ID NO. 6) may be similarly mutated to generate a herbicide resistant form of this enzyme.

A third resistant mutant has a G to A change at nucleotide 691 in the pWDC-2 sequence (SEQ ID No. 1); this plasmid is designated pAraC-3Ser. This mutation converts GGT codon for glycine at amino acid 221 SEQ ID No. 2) to an AGT codon for serine at the codon position adjacent to the mutation in pAraC-1. The corresponding glycine codon in the maize protox-1 sequence at nucleotide position 497-499 (SEQ ID NO. 5; amino acid position 167 of SEQ ID NO. 6) may be similarly mutated to generate a herbicide resistant form of this enzyme.

All the mutations described above result in a protox enzyme that is at least 10X more resistant to protox-inhibiting herbicide in the bacterial assay.

pAraC-2Cys, in the pFL61 vector, was deposited on November 14, 1994 under the designation pWDC-7 with the Agricultural Research Culture Collection and given the deposit designation NRRL #21339N.

EXAMPLE 25: Additional herbicide-resistant codon substitutions at positions identified in the random screen

The amino acids identified as herbicide resistance sites in the random screen are replaced by other amino acids and tested for function and for herbicide tolerance in the bacterial system. Oligonucleotide-directed mutagenesis of the Arabidopsis Protox-1 sequence is performed using the Transformer Site-Directed Mutagenesis Kit (Clontech, Palo Alto, CA). After amino acid changes are confirmed by sequence analysis, the mutated plasmids are transformed into SASX38 and plated on L-amp^{100} media to test for function and on various concentrations of protox-inhibiting herbicide to test for tolerance.

This procedure was applied to the alanine codon at nucleotides 688-690 and to the tyrosine codon at nucleotides 1306-1308 of the *Arabidopsis* protox sequence (SEQ ID No. 1). The results demonstrate that the alanine codon at nucleotides 688-690 can be changed to a codon for valine, threonine, leucine, or cysteine to yield a herbicide-resistant protox enzyme which retains function. The results further demonstrate that the tyrosine codon at nucleotides 1306-1308 can be changed to a codon for cysteine, isoleucine, leucine, valine or threonine to yield a herbicide-resistant protox enzyme which retains function.

EXAMPLE 26: Demonstration of resistant mutations' cross-tolerance to various protox-inhibiting compounds.

Resistant mutant plasmids, selected for resistance against a single herbicide, are tested against a spectrum of other protox-inhibiting compounds. The SASX38 strain containing the wild-type plasmid is plated on a range of concentrations of each compound to determine the lethal concentration for each one. Resistant mutant plasmids in SASX38 are plated and scored for the ability to survive on a concentration of each compound which is at least 10 fold higher than the concentration that is lethal to the SASX38 strain containing the wild-type plasmid.

Results from cross-tolerance testing show that each of the mutations identified confer tolerance to a variety of protox inhibiting compounds. In particular, the results show that 1) the AraC1-Val mutation confers resistance to protox inhibitors including,

but not necessarily limited to, those having the Formulae IV, XI, XIII, XIV, XV and XVII; 2) the AraC-2Cys mutation confers resistance to protox inhibitors including, but not necessarily limited to, those having Formulae XI, XIII, XV and XVII; 3) the MzC-1Val mutation confers resistance to protox inhibitors including, but not necessarily limited to, those having the Formulae XI, XII, XIII, XIV, XV, XVI and XVII; 4) the AraC-3Ser mutation confers resistance to protox inhibitors including, but not necessarily limited to, bifenox and those having the Formulae IV, XII, XIII, XIV, XV, and XVII.

EXAMPLE 27: Production of herbicide tolerant plants by overexpression of plant protox genes.

The Arabidopsis Protox-1 coding sequences from both the wild-type and the resistant mutant AraC-1Val genes are excised by partial EcoRI and XhoI digestion and cloned into the pCGN1761ENX plant expression plasmid. The expression cassettes containing 2X35S-Protox gene fusions areare excised by digestion with XbaI and cloned into the binary vector pCIB200. These binary protox plasmids are transformed by electroporation into *Agrobacterium* and then into *Arabidopsis* using the vacuum infiltration method (Bechtold et al., 1993). Transformants are selected on kanamycin, and T2 seed is generated from a number of independent lines. This seed is plated on GM media containing various concentrations of protox-inhibiting herbicide and scored for germination and survival. Multiple transgenic lines overexpressing either the wild type or the resistant mutant protox produce significant numbers of green seedlings on an herbicide concentration that is lethal to the empty vector control.

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Various modifications of the invention described herein will become apparent to those skilled in the art. Such modifications are intended to fall within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: CIBA-GEIGY AG
(B) STREET: Klybeckstr. 141
(C) CITY: Basel
(E) COUNTRY: Switzerland
(F) POSTAL CODE (ZIP): 4002
(G) TELEPHONE: +41 61 69 11 11
(H) TELEFAX: + 41 61 696 79 76
(I) TELEX: 962 991

(ii) TITLE OF INVENTION: MANIPULATION OF PROTOPORPHYRINOGEN OXIDASE ENZYME ACTIVITY IN EUKARYOTIC ORGANISMS

(iii) NUMBER OF SEQUENCES: 20

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1719 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 31..1644
(D) OTHER INFORMATION: /note= "Arabidopsis protox-1 cDNA; sequence from pWDC-2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
TGACAAAATT CCGAATTCTC TGCGATTTCC ATG GAG TTA TCT CTT CTC CGT CCG        54
                                 Met Glu Leu Ser Leu Leu Arg Pro
                                   1               5

ACG ACT CAA TCG CTT CTT CCG TCG TTT TCG AAG CCC AAT CTC CGA TTA        102
Thr Thr Gln Ser Leu Leu Pro Ser Phe Ser Lys Pro Asn Leu Arg Leu
     10                  15                  20

AAT GTT TAT AAG CCT CTT AGA CTC CGT TGT TCA GTG GCC GGT GGA CCA        150
Asn Val Tyr Lys Pro Leu Arg Leu Arg Cys Ser Val Ala Gly Gly Pro
 25                  30                  35                  40

ACC GTC GGA TCT TCA AAA ATC GAA GGC GGA GGA GGC ACC ACC ATC ACG        198
Thr Val Gly Ser Ser Lys Ile Glu Gly Gly Gly Gly Thr Thr Ile Thr
                 45                  50                  55

ACG GAT TGT GTG ATT GTC GGC GGA GGT ATT AGT GGT CTT TGC ATC GCT        246
Thr Asp Cys Val Ile Val Gly Gly Gly Ile Ser Gly Leu Cys Ile Ala
             60                  65                  70

CAG GCG CTT GCT ACT AAG CAT CCT GAT GCT GCT CCG AAT TTA ATT GTG        294
Gln Ala Leu Ala Thr Lys His Pro Asp Ala Ala Pro Asn Leu Ile Val
         75                  80                  85

ACC GAG GCT AAG GAT CGT GTT GGA GGC AAC ATT ATC ACT CGT GAA GAG        342
Thr Glu Ala Lys Asp Arg Val Gly Gly Asn Ile Ile Thr Arg Glu Glu
     90                  95                  100

AAT GGT TTT CTC TGG GAA GAA GGT CCC AAT AGT TTT CAA CCG TCT GAT        390
Asn Gly Phe Leu Trp Glu Glu Gly Pro Asn Ser Phe Gln Pro Ser Asp
105                 110                 115                 120

CCT ATG CTC ACT ATG GTG GTA GAT AGT GGT TTG AAG GAT GAT TTG GTG        438
Pro Met Leu Thr Met Val Val Asp Ser Gly Leu Lys Asp Asp Leu Val
                125                 130                 135

TTG GGA GAT CCT ACT GCG CCA AGG TTT GTG TTG TGG AAT GGG AAA TTG        486
Leu Gly Asp Pro Thr Ala Pro Arg Phe Val Leu Trp Asn Gly Lys Leu
            140                 145                 150

AGG CCG GTT CCA TCG AAG CTA ACA GAC TTA CCG TTC TTT GAT TTG ATG        534
Arg Pro Val Pro Ser Lys Leu Thr Asp Leu Pro Phe Phe Asp Leu Met
        155                 160                 165

AGT ATT GGT GGG AAG ATT AGA GCT GGT TTT GGT GCA CTT GGC ATT CGA        582
Ser Ile Gly Gly Lys Ile Arg Ala Gly Phe Gly Ala Leu Gly Ile Arg
    170                 175                 180

CCG TCA CCT CCA GGT CGT GAA GAA TCT GTG GAG GAG TTT GTA CGG CGT        630
Pro Ser Pro Pro Gly Arg Glu Glu Ser Val Glu Glu Phe Val Arg Arg
185                 190                 195                 200
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AAC CTC GGT GAT GAG GTT TTT GAG CGC CTG ATT GAA CCG TTT TGT TCA        678
Asn Leu Gly Asp Glu Val Phe Glu Arg Leu Ile Glu Pro Phe Cys Ser
                205                 210                 215

GGT GTT TAT GCT GGT GAT CCT TCA AAA CTG AGC ATG AAA GCA GCG TTT        726
Gly Val Tyr Ala Gly Asp Pro Ser Lys Leu Ser Met Lys Ala Ala Phe
            220                 225                 230

GGG AAG GTT TGG AAA CTA GAG CAA AAT GGT GGA AGC ATA ATA GGT GGT        774
Gly Lys Val Trp Lys Leu Glu Gln Asn Gly Gly Ser Ile Ile Gly Gly
        235                 240                 245

ACT TTT AAG GCA ATT CAG GAG AGG AAA AAC GCT CCC AAG GCA GAA CGA        822
Thr Phe Lys Ala Ile Gln Glu Arg Lys Asn Ala Pro Lys Ala Glu Arg
    250                 255                 260

GAC CCG CGC CTG CCA AAA CCA CAG GGC CAA ACA GTT GGT TCT TTC AGG        870
Asp Pro Arg Leu Pro Lys Pro Gln Gly Gln Thr Val Gly Ser Phe Arg
265                 270                 275                 280

AAG GGA CTT CGA ATG TTG CCA GAA GCA ATA TCT GCA AGA TTA GGT AGC        918
Lys Gly Leu Arg Met Leu Pro Glu Ala Ile Ser Ala Arg Leu Gly Ser
                285                 290                 295

AAA GTT AAG TTG TCT TGG AAG CTC TCA GGT ATC ACT AAG CTG GAG AGC        966
Lys Val Lys Leu Ser Trp Lys Leu Ser Gly Ile Thr Lys Leu Glu Ser
            300                 305                 310

GGA GGA TAC AAC TTA ACA TAT GAG ACT CCA GAT GGT TTA GTT TCC GTG       1014
Gly Gly Tyr Asn Leu Thr Tyr Glu Thr Pro Asp Gly Leu Val Ser Val
        315                 320                 325

CAG AGC AAA AGT GTT GTA ATG ACG GTG CCA TCT CAT GTT GCA AGT GGT       1062
Gln Ser Lys Ser Val Val Met Thr Val Pro Ser His Val Ala Ser Gly
    330                 335                 340

CTC TTG CGC CCT CTT TCT GAA TCT GCT GCA AAT GCA CTC TCA AAA CTA       1110
Leu Leu Arg Pro Leu Ser Glu Ser Ala Ala Asn Ala Leu Ser Lys Leu
345                 350                 355                 360

TAT TAC CCA CCA GTT GCA GCA GTA TCT ATC TCG TAC CCG AAA GAA GCA       1158
Tyr Tyr Pro Pro Val Ala Ala Val Ser Ile Ser Tyr Pro Lys Glu Ala
                365                 370                 375

ATC CGA ACA GAA TGT TTG ATA GAT GGT GAA CTA AAG GGT TTT GGG CAA       1206
Ile Arg Thr Glu Cys Leu Ile Asp Gly Glu Leu Lys Gly Phe Gly Gln
            380                 385                 390

TTG CAT CCA CGC ACG CAA GGA GTT GAA ACA TTA GGA ACT ATC TAC AGC       1254
Leu His Pro Arg Thr Gln Gly Val Glu Thr Leu Gly Thr Ile Tyr Ser
        395                 400                 405
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TCC TCA CTC TTT CCA AAT CGC GCA CCG CCC GGA AGA ATT TTG CTG TTG      1302
Ser Ser Leu Phe Pro Asn Arg Ala Pro Pro Gly Arg Ile Leu Leu Leu
    410                 415                 420

AAC TAC ATT GGC GGG TCT ACA AAC ACC GGA ATT CTG TCC AAG TCT GAA      1350
Asn Tyr Ile Gly Gly Ser Thr Asn Thr Gly Ile Leu Ser Lys Ser Glu
425                 430                 435                 440

GGT GAG TTA GTG GAA GCA GTT GAC AGA GAT TTG AGG AAA ATG CTA ATT      1398
Gly Glu Leu Val Glu Ala Val Asp Arg Asp Leu Arg Lys Met Leu Ile
                445                 450                 455

AAG CCT AAT TCG ACC GAT CCA CTT AAA TTA GGA GTT AGG GTA TGG CCT      1446
Lys Pro Asn Ser Thr Asp Pro Leu Lys Leu Gly Val Arg Val Trp Pro
            460                 465                 470

CAA GCC ATT CCT CAG TTT CTA GTT GGT CAC TTT GAT ATC CTT GAC ACG      1494
Gln Ala Ile Pro Gln Phe Leu Val Gly His Phe Asp Ile Leu Asp Thr
        475                 480                 485

GCT AAA TCA TCT CTA ACG TCT TCG GGC TAC GAA GGG CTA TTT TTG GGT      1542
Ala Lys Ser Ser Leu Thr Ser Ser Gly Tyr Glu Gly Leu Phe Leu Gly
    490                 495                 500

GGC AAT TAC GTC GCT GGT GTA GCC TTA GGC CGG TGT GTA GAA GGC GCA      1590
Gly Asn Tyr Val Ala Gly Val Ala Leu Gly Arg Cys Val Glu Gly Ala
505                 510                 515                 520

TAT GAA ACC GCG ATT GAG GTC AAC AAC TTC ATG TCA CGG TAC GCT TAC      1638
Tyr Glu Thr Ala Ile Glu Val Asn Asn Phe Met Ser Arg Tyr Ala Tyr
                525                 530                 535

AAG TAAATGTAAA ACATTAAATC TCCCAGCTTG CGTGAGTTTT ATTAAATATT           1691
Lys

TTGAGATATC CAAAAAAAAA AAAAAAAA                                       1719
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 537 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Glu Leu Ser Leu Leu Arg Pro Thr Thr Gln Ser Leu Leu Pro Ser
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1 5 10 15

Phe Ser Lys Pro Asn Leu Arg Leu Asn Val Tyr Lys Pro Leu Arg Leu
20 25 30

Arg Cys Ser Val Ala Gly Gly Pro Thr Val Gly Ser Ser Lys Ile Glu
35 40 45

Gly Gly Gly Gly Thr Thr Ile Thr Thr Asp Cys Val Ile Val Gly Gly
50 55 60

Gly Ile Ser Gly Leu Cys Ile Ala Gln Ala Leu Ala Thr Lys His Pro
65 70 75 80

Asp Ala Ala Pro Asn Leu Ile Val Thr Glu Ala Lys Asp Arg Val Gly
85 90 95

Gly Asn Ile Ile Thr Arg Glu Glu Asn Gly Phe Leu Trp Glu Glu Gly
100 105 110

Pro Asn Ser Phe Gln Pro Ser Asp Pro Met Leu Thr Met Val Val Asp
115 120 125

Ser Gly Leu Lys Asp Asp Leu Val Leu Gly Asp Pro Thr Ala Pro Arg
130 135 140

Phe Val Leu Trp Asn Gly Lys Leu Arg Pro Val Pro Ser Lys Leu Thr
145 150 155 160

Asp Leu Pro Phe Phe Asp Leu Met Ser Ile Gly Gly Lys Ile Arg Ala
165 170 175

Gly Phe Gly Ala Leu Gly Ile Arg Pro Ser Pro Pro Gly Arg Glu Glu
180 185 190

Ser Val Glu Glu Phe Val Arg Arg Asn Leu Gly Asp Glu Val Phe Glu
195 200 205

Arg Leu Ile Glu Pro Phe Cys Ser Gly Val Tyr Ala Gly Asp Pro Ser
210 215 220

Lys Leu Ser Met Lys Ala Ala Phe Gly Lys Val Trp Lys Leu Glu Gln
225 230 235 240

Asn Gly Gly Ser Ile Ile Gly Gly Thr Phe Lys Ala Ile Gln Glu Arg
245 250 255

Lys Asn Ala Pro Lys Ala Glu Arg Asp Pro Arg Leu Pro Lys Pro Gln
260 265 270

Gly Gln Thr Val Gly Ser Phe Arg Lys Gly Leu Arg Met Leu Pro Glu
275 280 285

Ala Ile Ser Ala Arg Leu Gly Ser Lys Val Lys Leu Ser Trp Lys Leu
290 295 300

Ser Gly Ile Thr Lys Leu Glu Ser Gly Gly Tyr Asn Leu Thr Tyr Glu
305 310 315 320

Thr Pro Asp Gly Leu Val Ser Val Gln Ser Lys Ser Val Val Met Thr
325 330 335

Val Pro Ser His Val Ala Ser Gly Leu Leu Arg Pro Leu Ser Glu Ser
340 345 350

Ala Ala Asn Ala Leu Ser Lys Leu Tyr Tyr Pro Pro Val Ala Ala Val
355 360 365

Ser Ile Ser Tyr Pro Lys Glu Ala Ile Arg Thr Glu Cys Leu Ile Asp
370 375 380

Gly Glu Leu Lys Gly Phe Gly Gln Leu His Pro Arg Thr Gln Gly Val
385 390 395 400

Glu Thr Leu Gly Thr Ile Tyr Ser Ser Ser Leu Phe Pro Asn Arg Ala
405 410 415

Pro Pro Gly Arg Ile Leu Leu Leu Asn Tyr Ile Gly Gly Ser Thr Asn
420 425 430

Thr Gly Ile Leu Ser Lys Ser Glu Gly Glu Leu Val Glu Ala Val Asp
435 440 445

Arg Asp Leu Arg Lys Met Leu Ile Lys Pro Asn Ser Thr Asp Pro Leu
450 455 460

Lys Leu Gly Val Arg Val Trp Pro Gln Ala Ile Pro Gln Phe Leu Val
465 470 475 480

Gly His Phe Asp Ile Leu Asp Thr Ala Lys Ser Ser Leu Thr Ser Ser
485 490 495

Gly Tyr Glu Gly Leu Phe Leu Gly Gly Asn Tyr Val Ala Gly Val Ala
500 505 510

Leu Gly Arg Cys Val Glu Gly Ala Tyr Glu Thr Ala Ile Glu Val Asn
515 520 525

Asn Phe Met Ser Arg Tyr Ala Tyr Lys
530 535

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1738 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 70..1596
(D) OTHER INFORMATION: /note= "Arabidopsis protox-2 cDNA; sequence from pWDC-1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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TTTTTTACTT ATTTCCGTCA CTGCTTTCGA CTGGTCAGAG ATTTTGACTC TGAATTGTTG      60

CAGATAGCA ATG GCG TCT GGA GCA GTA GCA GAT CAT CAA ATT GAA GCG          108
          Met Ala Ser Gly Ala Val Ala Asp His Gln Ile Glu Ala
            1               5                  10

GTT TCA GGA AAA AGA GTC GCA GTC GTA GGT GCA GGT GTA AGT GGA CTT       156
Val Ser Gly Lys Arg Val Ala Val Val Gly Ala Gly Val Ser Gly Leu
     15                  20                  25

GCG GCG GCT TAC AAG TTG AAA TCG AGG GGT TTG AAT GTG ACT GTG TTT       204
Ala Ala Ala Tyr Lys Leu Lys Ser Arg Gly Leu Asn Val Thr Val Phe
 30                  35                  40                  45

GAA GCT GAT GGA AGA GTA GGT GGG AAG TTG AGA AGT GTT ATG CAA AAT       252
Glu Ala Asp Gly Arg Val Gly Gly Lys Leu Arg Ser Val Met Gln Asn
                 50                  55                  60

GGT TTG ATT TGG GAT GAA GGA GCA AAC ACC ATG ACT GAG GCT GAG CCA       300
Gly Leu Ile Trp Asp Glu Gly Ala Asn Thr Met Thr Glu Ala Glu Pro
            65                  70                  75

GAA GTT GGG AGT TTA CTT GAT GAT CTT GGG CTT CGT GAG AAA CAA CAA       348
Glu Val Gly Ser Leu Leu Asp Asp Leu Gly Leu Arg Glu Lys Gln Gln
        80                  85                  90

TTT CCA ATT TCA CAG AAA AAG CGG TAT ATT GTG CGG AAT GGT GTA CCT       396
Phe Pro Ile Ser Gln Lys Lys Arg Tyr Ile Val Arg Asn Gly Val Pro
     95                  100                 105
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GTG ATG CTA CCT ACC AAT CCC ATA GAG CTG GTC ACA AGT AGT GTG CTC          444
Val Met Leu Pro Thr Asn Pro Ile Glu Leu Val Thr Ser Ser Val Leu
110                 115                 120                 125

TCT ACC CAA TCT AAG TTT CAA ATC TTG TTG GAA CCA TTT TTA TGG AAG          492
Ser Thr Gln Ser Lys Phe Gln Ile Leu Leu Glu Pro Phe Leu Trp Lys
                130                 135                 140

AAA AAG TCC TCA AAA GTC TCA GAT GCA TCT GCT GAA GAA AGT GTA AGC          540
Lys Lys Ser Ser Lys Val Ser Asp Ala Ser Ala Glu Glu Ser Val Ser
            145                 150                 155

GAG TTC TTT CAA CGC CAT TTT GGA CAA GAG GTT GTT GAC TAT CTC ATC          588
Glu Phe Phe Gln Arg His Phe Gly Gln Glu Val Val Asp Tyr Leu Ile
        160                 165                 170

GAC CCT TTT GTT GGT GGA ACA AGT GCT GCG GAC CCT GAT TCC CTT TCA          636
Asp Pro Phe Val Gly Gly Thr Ser Ala Ala Asp Pro Asp Ser Leu Ser
    175                 180                 185

ATG AAG CAT TCT TTC CCA GAT CTC TGG AAT GTA GAG AAA AGT TTT GGC          684
Met Lys His Ser Phe Pro Asp Leu Trp Asn Val Glu Lys Ser Phe Gly
190                 195                 200                 205

TCT ATT ATA GTC GGT GCA ATC AGA ACA AAG TTT GCT GCT AAA GGT GGT          732
Ser Ile Ile Val Gly Ala Ile Arg Thr Lys Phe Ala Ala Lys Gly Gly
                210                 215                 220

AAA AGT AGA GAC ACA AAG AGT TCT CCT GGC ACA AAA AAG GGT TCG CGT          780
Lys Ser Arg Asp Thr Lys Ser Ser Pro Gly Thr Lys Lys Gly Ser Arg
            225                 230                 235

GGG TCA TTC TCT TTT AAG GGG GGA ATG CAG ATT CTT CCT GAT ACG TTG          828
Gly Ser Phe Ser Phe Lys Gly Gly Met Gln Ile Leu Pro Asp Thr Leu
        240                 245                 250

TGC AAA AGT CTC TCA CAT GAT GAG ATC AAT TTA GAC TCC AAG GTA CTC          876
Cys Lys Ser Leu Ser His Asp Glu Ile Asn Leu Asp Ser Lys Val Leu
    255                 260                 265

TCT TTG TCT TAC AAT TCT GGA TCA AGA CAG GAG AAC TGG TCA TTA TCT          924
Ser Leu Ser Tyr Asn Ser Gly Ser Arg Gln Glu Asn Trp Ser Leu Ser
270                 275                 280                 285

TGT GTT TCG CAT AAT GAA ACG CAG AGA CAA AAC CCC CAT TAT GAT GCT          972
Cys Val Ser His Asn Glu Thr Gln Arg Gln Asn Pro His Tyr Asp Ala
                290                 295                 300

GTA ATT ATG ACG GCT CCT CTG TGC AAT GTG AAG GAG ATG AAG GTT ATG         1020
Val Ile Met Thr Ala Pro Leu Cys Asn Val Lys Glu Met Lys Val Met
            305                 310                 315
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AAA GGA GGA CAA CCC TTT CAG CTA AAC TTT CTC CCC GAG ATT AAT TAC      1068
Lys Gly Gly Gln Pro Phe Gln Leu Asn Phe Leu Pro Glu Ile Asn Tyr
        320                 325                 330

ATG CCC CTC TCG GTT TTA ATC ACC ACA TTC ACA AAG GAG AAA GTA AAG      1116
Met Pro Leu Ser Val Leu Ile Thr Thr Phe Thr Lys Glu Lys Val Lys
    335                 340                 345

AGA CCT CTT GAA GGC TTT GGG GTA CTC ATT CCA TCT AAG GAG CAA AAG      1164
Arg Pro Leu Glu Gly Phe Gly Val Leu Ile Pro Ser Lys Glu Gln Lys
350                 355                 360                 365

CAT GGT TTC AAA ACT CTA GGT ACA CTT TTT TCA TCA ATG ATG TTT CCA      1212
His Gly Phe Lys Thr Leu Gly Thr Leu Phe Ser Ser Met Met Phe Pro
                370                 375                 380

GAT CGT TCC CCT AGT GAC GTT CAT CTA TAT ACA ACT TTT ATT GGT GGG      1260
Asp Arg Ser Pro Ser Asp Val His Leu Tyr Thr Thr Phe Ile Gly Gly
            385                 390                 395

AGT AGG AAC CAG GAA CTA GCC AAA GCT TCC ACT GAC GAA TTA AAA CAA      1308
Ser Arg Asn Gln Glu Leu Ala Lys Ala Ser Thr Asp Glu Leu Lys Gln
        400                 405                 410

GTT GTG ACT TCT GAC CTT CAG CGA CTG TTG GGG GTT GAA GGT GAA CCC      1356
Val Val Thr Ser Asp Leu Gln Arg Leu Leu Gly Val Glu Gly Glu Pro
    415                 420                 425

GTG TCT GTC AAC CAT TAC TAT TGG AGG AAA GCA TTC CCG TTG TAT GAC      1404
Val Ser Val Asn His Tyr Tyr Trp Arg Lys Ala Phe Pro Leu Tyr Asp
430                 435                 440                 445

AGC AGC TAT GAC TCA GTC ATG GAA GCA ATT GAC AAG ATG GAG AAT GAT      1452
Ser Ser Tyr Asp Ser Val Met Glu Ala Ile Asp Lys Met Glu Asn Asp
                450                 455                 460

CTA CCT GGG TTC TTC TAT GCA GGT AAT CAT CGA GGG GGG CTC TCT GTT      1500
Leu Pro Gly Phe Phe Tyr Ala Gly Asn His Arg Gly Gly Leu Ser Val
            465                 470                 475

GGG AAA TCA ATA GCA TCA GGT TGC AAA GCA GCT GAC CTT GTG ATC TCA      1548
Gly Lys Ser Ile Ala Ser Gly Cys Lys Ala Ala Asp Leu Val Ile Ser
        480                 485                 490

TAC CTG GAG TCT TGC TCA AAT GAC AAG AAA CCA AAT GAC AGC TTA TAACATTGTC
1603
Tyr Leu Glu Ser Cys Ser Asn Asp Lys Lys Pro Asn Asp Ser Leu
    495                 500                 505

AAGGTTCGTC CCTTTTTATC ACTTACTTTG TAAACTTGTA AAATGCAACA AGCCGCCGTG    1663

CGATTAGCCA ACAACTCAGC AAAACCCAGA TTCTCATAAG GCTCACTAAT TCCAGAATAA    1723
```

ACTATTTATG TAAAA 1738

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 508 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Ala Ser Gly Ala Val Ala Asp His Gln Ile Glu Ala Val Ser Gly
 1               5                  10                  15

Lys Arg Val Ala Val Val Gly Ala Gly Val Ser Gly Leu Ala Ala Ala
            20                  25                  30

Tyr Lys Leu Lys Ser Arg Gly Leu Asn Val Thr Val Phe Glu Ala Asp
        35                  40                  45

Gly Arg Val Gly Gly Lys Leu Arg Ser Val Met Gln Asn Gly Leu Ile
    50                  55                  60

Trp Asp Glu Gly Ala Asn Thr Met Thr Glu Ala Glu Pro Glu Val Gly
65                  70                  75                  80

Ser Leu Leu Asp Asp Leu Gly Leu Arg Glu Lys Gln Gln Phe Pro Ile
                85                  90                  95

Ser Gln Lys Lys Arg Tyr Ile Val Arg Asn Gly Val Pro Val Met Leu
            100                 105                 110

Pro Thr Asn Pro Ile Glu Leu Val Thr Ser Ser Val Leu Ser Thr Gln
        115                 120                 125

Ser Lys Phe Gln Ile Leu Leu Glu Pro Phe Leu Trp Lys Lys Lys Ser
    130                 135                 140

Ser Lys Val Ser Asp Ala Ser Ala Glu Glu Ser Val Ser Glu Phe Phe
145                 150                 155                 160

Gln Arg His Phe Gly Gln Glu Val Val Asp Tyr Leu Ile Asp Pro Phe
                165                 170                 175

Val Gly Gly Thr Ser Ala Ala Asp Pro Asp Ser Leu Ser Met Lys His
            180                 185                 190

Ser Phe Pro Asp Leu Trp Asn Val Glu Lys Ser Phe Gly Ser Ile Ile
```

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        195                 200                 205

Val Gly Ala Ile Arg Thr Lys Phe Ala Ala Lys Gly Gly Lys Ser Arg
    210                 215                 220

Asp Thr Lys Ser Ser Pro Gly Thr Lys Lys Gly Ser Arg Gly Ser Phe
225                 230                 235                 240

Ser Phe Lys Gly Gly Met Gln Ile Leu Pro Asp Thr Leu Cys Lys Ser
                245                 250                 255

Leu Ser His Asp Glu Ile Asn Leu Asp Ser Lys Val Leu Ser Leu Ser
            260                 265                 270

Tyr Asn Ser Gly Ser Arg Gln Glu Asn Trp Ser Leu Ser Cys Val Ser
        275                 280                 285

His Asn Glu Thr Gln Arg Gln Asn Pro His Tyr Asp Ala Val Ile Met
    290                 295                 300

Thr Ala Pro Leu Cys Asn Val Lys Glu Met Lys Val Met Lys Gly Gly
305                 310                 315                 320

Gln Pro Phe Gln Leu Asn Phe Leu Pro Glu Ile Asn Tyr Met Pro Leu
                325                 330                 335

Ser Val Leu Ile Thr Thr Phe Thr Lys Glu Lys Val Lys Arg Pro Leu
            340                 345                 350

Glu Gly Phe Gly Val Leu Ile Pro Ser Lys Glu Gln Lys His Gly Phe
        355                 360                 365

Lys Thr Leu Gly Thr Leu Phe Ser Ser Met Met Phe Pro Asp Arg Ser
    370                 375                 380

Pro Ser Asp Val His Leu Tyr Thr Thr Phe Ile Gly Gly Ser Arg Asn
385                 390                 395                 400

Gln Glu Leu Ala Lys Ala Ser Thr Asp Glu Leu Lys Gln Val Val Thr
                405                 410                 415

Ser Asp Leu Gln Arg Leu Leu Gly Val Glu Gly Glu Pro Val Ser Val
            420                 425                 430

Asn His Tyr Tyr Trp Arg Lys Ala Phe Pro Leu Tyr Asp Ser Ser Tyr
        435                 440                 445

Asp Ser Val Met Glu Ala Ile Asp Lys Met Glu Asn Asp Leu Pro Gly
    450                 455                 460

Phe Phe Tyr Ala Gly Asn His Arg Gly Gly Leu Ser Val Gly Lys Ser
465                 470                 475                 480
```

Ile Ala Ser Gly Cys Lys Ala Ala Asp Leu Val Ile Ser Tyr Leu Glu
485 490 495

Ser Cys Ser Asn Asp Lys Lys Pro Asn Asp Ser Leu
500 505

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1698 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 2..1453
(D) OTHER INFORMATION: /note= "Maize protox-1 cDNA (not full-length); sequence from pWDC-4"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
G AAT TCG GCG GAC TGC GTC GTG GTG GGC GGA GGC ATC AGT GGC CTC          46
  Asn Ser Ala Asp Cys Val Val Val Gly Gly Gly Ile Ser Gly Leu
    1               5                  10                  15

TGC ACC GCG CAG GCG CTG GCC ACG CGG CAC GGC GTC GGG GAC GTG CTT        94
Cys Thr Ala Gln Ala Leu Ala Thr Arg His Gly Val Gly Asp Val Leu
                20                  25                  30

GTC ACG GAG GCC CGC GCC CGC CCC GGC GGC AAC ATT ACC ACC GTC GAG       142
Val Thr Glu Ala Arg Ala Arg Pro Gly Gly Asn Ile Thr Thr Val Glu
            35                  40                  45

CGC CCC GAG GAA GGG TAC CTC TGG GAG GAG GGT CCC AAC AGC TTC CAG       190
Arg Pro Glu Glu Gly Tyr Leu Trp Glu Glu Gly Pro Asn Ser Phe Gln
        50                  55                  60

CCC TCC GAC CCC GTT CTC ACC ATG GCC GTG GAC AGC GGA CTG AAG GAT       238
Pro Ser Asp Pro Val Leu Thr Met Ala Val Asp Ser Gly Leu Lys Asp
    65                  70                  75
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GAC TTG GTT TTT GGG GAC CCA AAC GCG CCG CGT TTC GTG CTG TGG GAG      286
Asp Leu Val Phe Gly Asp Pro Asn Ala Pro Arg Phe Val Leu Trp Glu
 80                  85                  90                  95

GGG AAG CTG AGG CCC GTG CCA TCC AAG CCC GCC GAC CTC CCG TTC TTC      334
Gly Lys Leu Arg Pro Val Pro Ser Lys Pro Ala Asp Leu Pro Phe Phe
            100                 105                 110

GAT CTC ATG AGC ATC CCA GGG AAG CTC AGG GCC GGT CTA GGC GCG CTT      382
Asp Leu Met Ser Ile Pro Gly Lys Leu Arg Ala Gly Leu Gly Ala Leu
        115                 120                 125

GGC ATC CGC CCG CCT CCT CCA GGC CGC GAA GAG TCA GTG GAG GAG TTC      430
Gly Ile Arg Pro Pro Pro Pro Gly Arg Glu Glu Ser Val Glu Glu Phe
    130                 135                 140

GTG CGC CGC AAC CTC GGT GCT GAG GTC TTT GAG CGC CTC ATT GAG CCT      478
Val Arg Arg Asn Leu Gly Ala Glu Val Phe Glu Arg Leu Ile Glu Pro
145                 150                 155

TTC TGC TCA GGT GTC TAT GCT GGT GAT CCT TCT AAG CTC AGC ATG AAG      526
Phe Cys Ser Gly Val Tyr Ala Gly Asp Pro Ser Lys Leu Ser Met Lys
160                 165                 170                 175

GCT GCA TTT GGG AAG GTT TGG CGG TTG GAA GAA ACT GGA GGT AGT ATT      574
Ala Ala Phe Gly Lys Val Trp Arg Leu Glu Glu Thr Gly Gly Ser Ile
            180                 185                 190

ATT GGT GGA ACC ATC AAG ACA ATT CAG GAG AGG AGC AAG AAT CCA AAA      622
Ile Gly Gly Thr Ile Lys Thr Ile Gln Glu Arg Ser Lys Asn Pro Lys
        195                 200                 205

CCA CCG AGG GAT GCC CGC CTT CCG AAG CCA AAA GGG CAG ACA GTT GCA      670
Pro Pro Arg Asp Ala Arg Leu Pro Lys Pro Lys Gly Gln Thr Val Ala
    210                 215                 220

TCT TTC AGG AAG GGT CTT GCC ATG CTT CCA AAT GCC ATT ACA TCC AGC      718
Ser Phe Arg Lys Gly Leu Ala Met Leu Pro Asn Ala Ile Thr Ser Ser
225                 230                 235

TTG GGT AGT AAA GTC AAA CTA TCA TGG AAA CTC ACG AGC ATT ACA AAA      766
Leu Gly Ser Lys Val Lys Leu Ser Trp Lys Leu Thr Ser Ile Thr Lys
240                 245                 250                 255

TCA GAT GAC AAG GGA TAT GTT TTG GAG TAT GAA ACG CCA GAA GGG GTT      814
Ser Asp Asp Lys Gly Tyr Val Leu Glu Tyr Glu Thr Pro Glu Gly Val
            260                 265                 270

GTT TCG GTG CAG GCT AAA AGT GTT ATC ATG ACT ATT CCA TCA TAT GTT      862
Val Ser Val Gln Ala Lys Ser Val Ile Met Thr Ile Pro Ser Tyr Val
        275                 280                 285
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GCT AGC AAC ATT TTG CGT CCA CTT TCA AGC GAT GCT GCA GAT GCT CTA       910
Ala Ser Asn Ile Leu Arg Pro Leu Ser Ser Asp Ala Ala Asp Ala Leu
        290                 295                 300

TCA AGA TTC TAT TAT CCA CCG GTT GCT GCT GTA ACT GTT TCG TAT CCA       958
Ser Arg Phe Tyr Tyr Pro Pro Val Ala Ala Val Thr Val Ser Tyr Pro
    305                 310                 315

AAG GAA GCA ATT AGA AAA GAA TGC TTA ATT GAT GGG GAA CTC CAG GGC      1006
Lys Glu Ala Ile Arg Lys Glu Cys Leu Ile Asp Gly Glu Leu Gln Gly
320                 325                 330                 335

TTT GGC CAG TTG CAT CCA CGT AGT CAA GGA GTT GAG ACA TTA GGA ACA      1054
Phe Gly Gln Leu His Pro Arg Ser Gln Gly Val Glu Thr Leu Gly Thr
                340                 345                 350

ATA TAC AGT TCC TCA CTC TTT CCA AAT CGT GCT CCT GAC GGT AGG GTG      1102
Ile Tyr Ser Ser Ser Leu Phe Pro Asn Arg Ala Pro Asp Gly Arg Val
            355                 360                 365

TTA CTT CTA AAC TAC ATA GGA GGT GCT ACA AAC ACA GGA ATT GTT TCC      1150
Leu Leu Leu Asn Tyr Ile Gly Gly Ala Thr Asn Thr Gly Ile Val Ser
        370                 375                 380

AAG ACT GAA AGT GAG CTG GTC GAA GCA GTT GAC CGT GAC CTC CGA AAA      1198
Lys Thr Glu Ser Glu Leu Val Glu Ala Val Asp Arg Asp Leu Arg Lys
    385                 390                 395

ATG CTT ATA AAT TCT ACA GCA GTG GAC CCT TTA GTC CTT GGT GTT CGA      1246
Met Leu Ile Asn Ser Thr Ala Val Asp Pro Leu Val Leu Gly Val Arg
400                 405                 410                 415

GTT TGG CCA CAA GCC ATA CCT CAG TTC CTG GTA GGA CAT CTT GAT CTT      1294
Val Trp Pro Gln Ala Ile Pro Gln Phe Leu Val Gly His Leu Asp Leu
                420                 425                 430

CTG GAA GCC GCA AAA GCT GCC CTG GAC CGA GGT GGC TAC GAT GGG CTG      1342
Leu Glu Ala Ala Lys Ala Ala Leu Asp Arg Gly Gly Tyr Asp Gly Leu
            435                 440                 445

TTC CTA GGA GGG AAC TAT GTT GCA GGA GTT GCC CTG GGC AGA TGC GTT      1390
Phe Leu Gly Gly Asn Tyr Val Ala Gly Val Ala Leu Gly Arg Cys Val
        450                 455                 460

GAG GGC GCG TAT GAA AGT GCC TCG CAA ATA TCT GAC TTC TTG ACC AAG      1438
Glu Gly Ala Tyr Glu Ser Ala Ser Gln Ile Ser Asp Phe Leu Thr Lys
    465                 470                 475

TAT GCC TAC AAG TGATGAAAGA AGTGGAGCGC TACTTGTTAA TCGTTTATGT         1490
Tyr Ala Tyr Lys
480
```

TGCATAGATG AGGTGCCTCC GGGGAAAAAA AAGCTTGAAT AGTATTTTTT ATTCTTATTT 1550

TGTAAATTGC ATTTCTGTTC TTTTTTCTAT CAGTAATTAG TTATATTTTA GTTCTGTAGG 1610

AGATTGTTCT GTTCACTGCC CTTCAAAAGA AATTTTATTT TTCATTCTTT TATGAGAGCT 1670

GTGCTACTTA AAAAAAAAAA AAAAAAAA 1698

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 483 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Asn Ser Ala Asp Cys Val Val Val Gly Gly Gly Ile Ser Gly Leu Cys
1 5 10 15

Thr Ala Gln Ala Leu Ala Thr Arg His Gly Val Gly Asp Val Leu Val
20 25 30

Thr Glu Ala Arg Ala Arg Pro Gly Gly Asn Ile Thr Thr Val Glu Arg
35 40 45

Pro Glu Glu Gly Tyr Leu Trp Glu Glu Gly Pro Asn Ser Phe Gln Pro
50 55 60

Ser Asp Pro Val Leu Thr Met Ala Val Asp Ser Gly Leu Lys Asp Asp
65 70 75 80

Leu Val Phe Gly Asp Pro Asn Ala Pro Arg Phe Val Leu Trp Glu Gly
85 90 95

Lys Leu Arg Pro Val Pro Ser Lys Pro Ala Asp Leu Pro Phe Phe Asp
100 105 110

Leu Met Ser Ile Pro Gly Lys Leu Arg Ala Gly Leu Gly Ala Leu Gly
115 120 125

Ile Arg Pro Pro Pro Pro Gly Arg Glu Glu Ser Val Glu Glu Phe Val
130 135 140

Arg Arg Asn Leu Gly Ala Glu Val Phe Glu Arg Leu Ile Glu Pro Phe
145 150 155 160

Cys Ser Gly Val Tyr Ala Gly Asp Pro Ser Lys Leu Ser Met Lys Ala
165 170 175

Ala Phe Gly Lys Val Trp Arg Leu Glu Glu Thr Gly Gly Ser Ile Ile
180 185 190

Gly Gly Thr Ile Lys Thr Ile Gln Glu Arg Ser Lys Asn Pro Lys Pro
195 200 205

Pro Arg Asp Ala Arg Leu Pro Lys Pro Lys Gly Gln Thr Val Ala Ser
210 215 220

Phe Arg Lys Gly Leu Ala Met Leu Pro Asn Ala Ile Thr Ser Ser Leu
225 230 235 240

Gly Ser Lys Val Lys Leu Ser Trp Lys Leu Thr Ser Ile Thr Lys Ser
245 250 255

Asp Asp Lys Gly Tyr Val Leu Glu Tyr Glu Thr Pro Glu Gly Val Val
260 265 270

Ser Val Gln Ala Lys Ser Val Ile Met Thr Ile Pro Ser Tyr Val Ala
275 280 285

Ser Asn Ile Leu Arg Pro Leu Ser Ser Asp Ala Ala Asp Ala Leu Ser
290 295 300

Arg Phe Tyr Tyr Pro Pro Val Ala Ala Val Thr Val Ser Tyr Pro Lys
305 310 315 320

Glu Ala Ile Arg Lys Glu Cys Leu Ile Asp Gly Glu Leu Gln Gly Phe
325 330 335

Gly Gln Leu His Pro Arg Ser Gln Gly Val Glu Thr Leu Gly Thr Ile
340 345 350

Tyr Ser Ser Ser Leu Phe Pro Asn Arg Ala Pro Asp Gly Arg Val Leu
355 360 365

Leu Leu Asn Tyr Ile Gly Gly Ala Thr Asn Thr Gly Ile Val Ser Lys
370 375 380

Thr Glu Ser Glu Leu Val Glu Ala Val Asp Arg Asp Leu Arg Lys Met
385 390 395 400

Leu Ile Asn Ser Thr Ala Val Asp Pro Leu Val Leu Gly Val Arg Val
405 410 415

Trp Pro Gln Ala Ile Pro Gln Phe Leu Val Gly His Leu Asp Leu Leu
420 425 430

Glu Ala Ala Lys Ala Ala Leu Asp Arg Gly Gly Tyr Asp Gly Leu Phe
435 440 445

```
Leu Gly Gly Asn Tyr Val Ala Gly Val Ala Leu Gly Arg Cys Val Glu
    450                 455                 460

Gly Ala Tyr Glu Ser Ala Ser Gln Ile Ser Asp Phe Leu Thr Lys Tyr
465                 470                 475                 480

Ala Tyr Lys
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2061 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 64..1698
(D) OTHER INFORMATION: /note= "Maize protox-2 cDNA; sequence from pWDC-3"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
CTCTCCTACC TCCACCTCCA CGACAACAAG CAAATCCCCA TCCAGTTCCA AACCCTAACT      60

CAA ATG CTC GCT TTG ACT GCC TCA GCC TCA TCC GCT TCG TCC CAT CCT      108
    Met Leu Ala Leu Thr Ala Ser Ala Ser Ser Ala Ser Ser His Pro
      1               5                  10                  15

TAT CGC CAC GCC TCC GCG CAC ACT CGT CGC CCC CGC CTA CGT GCG GTC      156
Tyr Arg His Ala Ser Ala His Thr Arg Arg Pro Arg Leu Arg Ala Val
                20                  25                  30

CTC GCG ATG GCG GGC TCC GAC GAC CCC CGT GCA GCG CCC GCC AGA TCG      204
Leu Ala Met Ala Gly Ser Asp Asp Pro Arg Ala Ala Pro Ala Arg Ser
            35                  40                  45

GTC GCC GTC GTC GGC GCC GGG GTC AGC GGG CTC GCG GCG GCG TAC AGG      252
Val Ala Val Val Gly Ala Gly Val Ser Gly Leu Ala Ala Ala Tyr Arg
        50                  55                  60
```

```
CTC AGA CAG AGC GGC GTG AAC GTA ACG GTG TTC GAA GCG GCC GAC AGG      300
Leu Arg Gln Ser Gly Val Asn Val Thr Val Phe Glu Ala Ala Asp Arg
 65                  70                  75

GCG GGA GGA AAG ATA CGG ACC AAT TCC GAG GGC GGG TTT GTC TGG GAT      348
Ala Gly Gly Lys Ile Arg Thr Asn Ser Glu Gly Gly Phe Val Trp Asp
 80                  85                  90                  95

GAA GGA GCT AAC ACC ATG ACA GAA GGT GAA TGG GAG GCC AGT AGA CTG      396
Glu Gly Ala Asn Thr Met Thr Glu Gly Glu Trp Glu Ala Ser Arg Leu
                100                 105                 110

ATT GAT GAT CTT GGT CTA CAA GAC AAA CAG CAG TAT CCT AAC TCC CAA      444
Ile Asp Asp Leu Gly Leu Gln Asp Lys Gln Gln Tyr Pro Asn Ser Gln
            115                 120                 125

CAC AAG CGT TAC ATT GTC AAA GAT GGA GCA CCA GCA CTG ATT CCT TCG      492
His Lys Arg Tyr Ile Val Lys Asp Gly Ala Pro Ala Leu Ile Pro Ser
        130                 135                 140

GAT CCC ATT TCG CTA ATG AAA AGC AGT GTT CTT TCG ACA AAA TCA AAG      540
Asp Pro Ile Ser Leu Met Lys Ser Ser Val Leu Ser Thr Lys Ser Lys
    145                 150                 155

ATT GCG TTA TTT TTT GAA CCA TTT CTC TAC AAG AAA GCT AAC ACA AGA      588
Ile Ala Leu Phe Phe Glu Pro Phe Leu Tyr Lys Lys Ala Asn Thr Arg
160                 165                 170                 175

AAC TCT GGA AAA GTG TCT GAG GAG CAC TTG AGT GAG AGT GTT GGG AGC      636
Asn Ser Gly Lys Val Ser Glu Glu His Leu Ser Glu Ser Val Gly Ser
                180                 185                 190

TTC TGT GAA CGC CAC TTT GGA AGA GAA GTT GTT GAC TAT TTT GTT GAT      684
Phe Cys Glu Arg His Phe Gly Arg Glu Val Val Asp Tyr Phe Val Asp
            195                 200                 205

CCA TTT GTA GCT GGA ACA AGT GCA GGA GAT CCA GAG TCA CTA TCT ATT      732
Pro Phe Val Ala Gly Thr Ser Ala Gly Asp Pro Glu Ser Leu Ser Ile
        210                 215                 220

CGT CAT GCA TTC CCA GCA TTG TGG AAT TTG GAA AGA AAG TAT GGT TCA      780
Arg His Ala Phe Pro Ala Leu Trp Asn Leu Glu Arg Lys Tyr Gly Ser
    225                 230                 235

GTT ATT GTT GGT GCC ATC TTG TCT AAG CTA GCA GCT AAA GGT GAT CCA      828
Val Ile Val Gly Ala Ile Leu Ser Lys Leu Ala Ala Lys Gly Asp Pro
240                 245                 250                 255

GTA AAG ACA AGA CAT GAT TCA TCA GGG AAA AGA AGG AAT AGA CGA GTG      876
Val Lys Thr Arg His Asp Ser Ser Gly Lys Arg Arg Asn Arg Arg Val
                260                 265                 270
```

```
TCG TTT TCA TTT CAT GGT GGA ATG CAG TCA CTA ATA AAT GCA CTT CAC        924
Ser Phe Ser Phe His Gly Gly Met Gln Ser Leu Ile Asn Ala Leu His
            275                 280                 285

AAT GAA GTT GGA GAT GAT AAT GTG AAG CTT GGT ACA GAA GTG TTG TCA        972
Asn Glu Val Gly Asp Asp Asn Val Lys Leu Gly Thr Glu Val Leu Ser
        290                 295                 300

TTG GCA TGT ACA TTT GAT GGA GTT CCT GCA CTA GGC AGG TGG TCA ATT       1020
Leu Ala Cys Thr Phe Asp Gly Val Pro Ala Leu Gly Arg Trp Ser Ile
    305                 310                 315

TCT GTT GAT TCG AAG GAT AGC GGT GAC AAG GAC CTT GCT AGT AAC CAA       1068
Ser Val Asp Ser Lys Asp Ser Gly Asp Lys Asp Leu Ala Ser Asn Gln
320                 325                 330                 335

ACC TTT GAT GCT GTT ATA ATG ACA GCT CCA TTG TCA AAT GTC CGG AGG       1116
Thr Phe Asp Ala Val Ile Met Thr Ala Pro Leu Ser Asn Val Arg Arg
                340                 345                 350

ATG AAG TTC ACC AAA GGT GGA GCT CCG GTT GTT CTT GAC TTT CTT CCT       1164
Met Lys Phe Thr Lys Gly Gly Ala Pro Val Val Leu Asp Phe Leu Pro
            355                 360                 365

AAG ATG GAT TAT CTA CCA CTA TCT CTC ATG GTG ACT GCT TTT AAG AAG       1212
Lys Met Asp Tyr Leu Pro Leu Ser Leu Met Val Thr Ala Phe Lys Lys
        370                 375                 380

GAT GAT GTC AAG AAA CCT CTG GAA GGA TTT GGG GTC TTA ATA CCT TAC       1260
Asp Asp Val Lys Lys Pro Leu Glu Gly Phe Gly Val Leu Ile Pro Tyr
    385                 390                 395

AAG GAA CAG CAA AAA CAT GGT CTG AAA ACC CTT GGG ACT CTC TTT TCC       1308
Lys Glu Gln Gln Lys His Gly Leu Lys Thr Leu Gly Thr Leu Phe Ser
400                 405                 410                 415

TCA ATG ATG TTC CCA GAT CGA GCT CCT GAT GAC CAA TAT TTA TAT ACA       1356
Ser Met Met Phe Pro Asp Arg Ala Pro Asp Asp Gln Tyr Leu Tyr Thr
                420                 425                 430

ACA TTT GTT GGG GGT AGC CAC AAT AGA GAT CTT GCT GGA GCT CCA ACG       1404
Thr Phe Val Gly Gly Ser His Asn Arg Asp Leu Ala Gly Ala Pro Thr
            435                 440                 445

TCT ATT CTG AAA CAA CTT GTG ACC TCT GAC CTT AAA AAA CTC TTG GGC       1452
Ser Ile Leu Lys Gln Leu Val Thr Ser Asp Leu Lys Lys Leu Leu Gly
        450                 455                 460

GTA GAG GGG CAA CCA ACT TTT GTC AAG CAT GTA TAC TGG GGA AAT GCT       1500
Val Glu Gly Gln Pro Thr Phe Val Lys His Val Tyr Trp Gly Asn Ala
    465                 470                 475
```

```
TTT CCT TTG TAT GGC CAT GAT TAT AGT TCT GTA TTG GAA GCT ATA GAA      1548
Phe Pro Leu Tyr Gly His Asp Tyr Ser Ser Val Leu Glu Ala Ile Glu
480                 485                 490                 495

AAG ATG GAG AAA AAC CTT CCA GGG TTC TTC TAC GCA GGA AAT AGC AAG      1596
Lys Met Glu Lys Asn Leu Pro Gly Phe Phe Tyr Ala Gly Asn Ser Lys
                500                 505                 510

GAT GGG CTT GCT GTT GGA AGT GTT ATA GCT TCA GGA AGC AAG GCT GCT      1644
Asp Gly Leu Ala Val Gly Ser Val Ile Ala Ser Gly Ser Lys Ala Ala
            515                 520                 525

GAC CTT GCA ATC TCA TAT CTT GAA TCT CAC ACC AAG CAT AAT AAT TCA      1692
Asp Leu Ala Ile Ser Tyr Leu Glu Ser His Thr Lys His Asn Asn Ser
        530                 535                 540

CAT TGAAAGTGTC TGACCTATCC TCTAGCAGTT GTCGACAAAT TTCTCCAGTT           1745
His
    545

CATGTACAGT AGAAACCGAT GCGTTGCAGT TTCAGAACAT CTTCACTTCT TCAGATATTA    1805

ACCCTTCGTT GAACATCCAC CAGAAAGGTA GTCACATGTG TAAGTGGGAA AATGAGGTTA    1865

AAAACTATTA TGGCGGCCGA AATGTTCCTT TTTGTTTTCC TCACAAGTGG CCTACGACAC    1925

TTGATGTTGG AAATACATTT AAATTTGTTG AATTGTTTGA GAACACATGC GTGACGTGTA    1985

ATATTTGCCT ATTGTGATTT TAGCAGTAGT CTTGGCCAGA TTATGCTTTA CGCCTTTAAA    2045

AAAAAAAAAA AAAAAA                                                   2061
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 544 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Met Leu Ala Leu Thr Ala Ser Ala Ser Ser Ala Ser Ser His Pro Tyr
 1               5                  10                  15

Arg His Ala Ser Ala His Thr Arg Arg Pro Arg Leu Arg Ala Val Leu
            20                  25                  30

Ala Met Ala Gly Ser Asp Asp Pro Arg Ala Ala Pro Ala Arg Ser Val
        35                  40                  45
```

```
Ala Val Val Gly Ala Gly Val Ser Gly Leu Ala Ala Ala Tyr Arg Leu
     50                  55                  60

Arg Gln Ser Gly Val Asn Val Thr Val Phe Glu Ala Ala Asp Arg Ala
 65                  70                  75                  80

Gly Gly Lys Ile Arg Thr Asn Ser Glu Gly Gly Phe Val Trp Asp Glu
                 85                  90                  95

Gly Ala Asn Thr Met Thr Glu Gly Glu Trp Glu Ala Ser Arg Leu Ile
            100                 105                 110

Asp Asp Leu Gly Leu Gln Asp Lys Gln Gln Tyr Pro Asn Ser Gln His
        115                 120                 125

Lys Arg Tyr Ile Val Lys Asp Gly Ala Pro Ala Leu Ile Pro Ser Asp
    130                 135                 140

Pro Ile Ser Leu Met Lys Ser Ser Val Leu Ser Thr Lys Ser Lys Ile
145                 150                 155                 160

Ala Leu Phe Phe Glu Pro Phe Leu Tyr Lys Lys Ala Asn Thr Arg Asn
                165                 170                 175

Ser Gly Lys Val Ser Glu Glu His Leu Ser Glu Ser Val Gly Ser Phe
            180                 185                 190

Cys Glu Arg His Phe Gly Arg Glu Val Val Asp Tyr Phe Val Asp Pro
        195                 200                 205

Phe Val Ala Gly Thr Ser Ala Gly Asp Pro Glu Ser Leu Ser Ile Arg
    210                 215                 220

His Ala Phe Pro Ala Leu Trp Asn Leu Glu Arg Lys Tyr Gly Ser Val
225                 230                 235                 240

Ile Val Gly Ala Ile Leu Ser Lys Leu Ala Ala Lys Gly Asp Pro Val
                245                 250                 255

Lys Thr Arg His Asp Ser Ser Gly Lys Arg Arg Asn Arg Arg Val Ser
            260                 265                 270

Phe Ser Phe His Gly Gly Met Gln Ser Leu Ile Asn Ala Leu His Asn
        275                 280                 285

Glu Val Gly Asp Asp Asn Val Lys Leu Gly Thr Glu Val Leu Ser Leu
    290                 295                 300

Ala Cys Thr Phe Asp Gly Val Pro Ala Leu Gly Arg Trp Ser Ile Ser
305                 310                 315                 320
```

```
Val Asp Ser Lys Asp Ser Gly Asp Lys Asp Leu Ala Ser Asn Gln Thr
                325                 330                 335

Phe Asp Ala Val Ile Met Thr Ala Pro Leu Ser Asn Val Arg Arg Met
            340                 345                 350

Lys Phe Thr Lys Gly Gly Ala Pro Val Val Leu Asp Phe Leu Pro Lys
        355                 360                 365

Met Asp Tyr Leu Pro Leu Ser Leu Met Val Thr Ala Phe Lys Lys Asp
    370                 375                 380

Asp Val Lys Lys Pro Leu Glu Gly Phe Gly Val Leu Ile Pro Tyr Lys
385                 390                 395                 400

Glu Gln Gln Lys His Gly Leu Lys Thr Leu Gly Thr Leu Phe Ser Ser
                405                 410                 415

Met Met Phe Pro Asp Arg Ala Pro Asp Asp Gln Tyr Leu Tyr Thr Thr
            420                 425                 430

Phe Val Gly Gly Ser His Asn Arg Asp Leu Ala Gly Ala Pro Thr Ser
        435                 440                 445

Ile Leu Lys Gln Leu Val Thr Ser Asp Leu Lys Lys Leu Leu Gly Val
    450                 455                 460

Glu Gly Gln Pro Thr Phe Val Lys His Val Tyr Trp Gly Asn Ala Phe
465                 470                 475                 480

Pro Leu Tyr Gly His Asp Tyr Ser Ser Val Leu Glu Ala Ile Glu Lys
                485                 490                 495

Met Glu Lys Asn Leu Pro Gly Phe Phe Tyr Ala Gly Asn Ser Lys Asp
            500                 505                 510

Gly Leu Ala Val Gly Ser Val Ile Ala Ser Gly Ser Lys Ala Ala Asp
        515                 520                 525

Leu Ala Ile Ser Tyr Leu Glu Ser His Thr Lys His Asn Asn Ser His
    530                 535                 540
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1697 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 29..1501
(D) OTHER INFORMATION: /note= "yeast protox-3 cDNA; sequence from pWDC-5"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
TTGGCATTTG CCTTGAACCA ACAATTCT ATG TCA ATT GCA ATT TGT GGA GGA          52
                                Met Ser Ile Ala Ile Cys Gly Gly
                                  1               5

GGT ATA GCT GGT CTT AGT ACA GCA TTT TAT CTT GCT AGA TTG ATT CCA         100
Gly Ile Ala Gly Leu Ser Thr Ala Phe Tyr Leu Ala Arg Leu Ile Pro
     10                  15                  20

AAA TGT ACT ATT GAT TTG TAC GAA AAA GGT CCT CGT TTA GGT GGA TGG         148
Lys Cys Thr Ile Asp Leu Tyr Glu Lys Gly Pro Arg Leu Gly Gly Trp
 25                  30                  35                  40

CTT CAG TCG GTC AAA ATC CCG TGT GCA GAT TCT CCA ACA GGA ACG GTT         196
Leu Gln Ser Val Lys Ile Pro Cys Ala Asp Ser Pro Thr Gly Thr Val
                 45                  50                  55

TTG TTT GAG CAA GGT CCT AGA ACT CTT CGT CCT GCT GGG GTT GCT GGC         244
Leu Phe Glu Gln Gly Pro Arg Thr Leu Arg Pro Ala Gly Val Ala Gly
             60                  65                  70

TTA GCA AAC TTA GAT TTA ATT AGC AAG TTG GGC ATC GAA GAC AAG TTG         292
Leu Ala Asn Leu Asp Leu Ile Ser Lys Leu Gly Ile Glu Asp Lys Leu
         75                  80                  85

TTA AGG ATT TCG AGC AAT TCT CCC AGC GCA AAA AAC CGA TAT ATT TAT         340
Leu Arg Ile Ser Ser Asn Ser Pro Ser Ala Lys Asn Arg Tyr Ile Tyr
     90                  95                 100

TAC CCA GAT CGC TTA AAT GAA ATT CCT TCA AGC ATT TTA GGG AGT ATA         388
Tyr Pro Asp Arg Leu Asn Glu Ile Pro Ser Ser Ile Leu Gly Ser Ile
105                 110                 115                 120

AAG TCG ATT ATG CAG CCT GCT TTG CGT CCG ATG CCT TTG GCT ATG ATG         436
Lys Ser Ile Met Gln Pro Ala Leu Arg Pro Met Pro Leu Ala Met Met
```

```
                  125                 130                 135

CTT GAG CCC TTT CGT AAA AGT AAG CGA GAT TCG ACA GAT GAA AGC GTG      484
Leu Glu Pro Phe Arg Lys Ser Lys Arg Asp Ser Thr Asp Glu Ser Val
            140                 145                 150

GGT TCA TTT ATG AGA AGA AGA TTT GGT AAA AAC GTT ACG GAT AGA GTT      532
Gly Ser Phe Met Arg Arg Arg Phe Gly Lys Asn Val Thr Asp Arg Val
        155                 160                 165

ATG AGT GCA ATG ATA AAT GGT ATT TAT GCT GGT GAT TTG AAT GAT TTG      580
Met Ser Ala Met Ile Asn Gly Ile Tyr Ala Gly Asp Leu Asn Asp Leu
    170                 175                 180

TCT ATG CAT TCT AGC ATG TTT GGA TTT TTA GCG AAG ATT GAA AAA AAG      628
Ser Met His Ser Ser Met Phe Gly Phe Leu Ala Lys Ile Glu Lys Lys
185                 190                 195                 200

TAT GGA AAC ATT ACT TTG GGA TTA ATT AGA GCT CTT CTT GCA CGT GAA      676
Tyr Gly Asn Ile Thr Leu Gly Leu Ile Arg Ala Leu Leu Ala Arg Glu
                205                 210                 215

ATA TTA TCT CCT GCT GAG AAA GCT TTG GAA AGC AGC ACT ACT CGC AGA      724
Ile Leu Ser Pro Ala Glu Lys Ala Leu Glu Ser Ser Thr Thr Arg Arg
            220                 225                 230

GCC AAA AAC AGC AGA GCT GTC AAA CAG TAT GAA ATC GAC AAG TAT GTT      772
Ala Lys Asn Ser Arg Ala Val Lys Gln Tyr Glu Ile Asp Lys Tyr Val
        235                 240                 245

GCT TTC AAG GAA GGG ATT GAG ACT ATT ACA TTG TCA ATA GCA GAT GAA      820
Ala Phe Lys Glu Gly Ile Glu Thr Ile Thr Leu Ser Ile Ala Asp Glu
    250                 255                 260

TTA AAA AAA ATG CCG AAT GTC AAG ATA CAT CTA AAC AAA CCG GCC CAA      868
Leu Lys Lys Met Pro Asn Val Lys Ile His Leu Asn Lys Pro Ala Gln
265                 270                 275                 280

ACT TTG GTT CCA CAT AAA ACT CAG TCT CTT GTA GAC GTC AAT GGT CAA      916
Thr Leu Val Pro His Lys Thr Gln Ser Leu Val Asp Val Asn Gly Gln
                285                 290                 295

GCT TAC GAG TAT GTT GTG TTT GCA AAC TCT TCT CGC AAT TTA GAG AAT      964
Ala Tyr Glu Tyr Val Val Phe Ala Asn Ser Ser Arg Asn Leu Glu Asn
            300                 305                 310

CTA ATA TCT TGT CCT AAA ATG GAA ACT CCG ACG TCG AGT GTT TAT GTC     1012
Leu Ile Ser Cys Pro Lys Met Glu Thr Pro Thr Ser Ser Val Tyr Val
        315                 320                 325

GTC AAC GTT TAT TAT AAG GAC CCT AAT GTT CTT CCA ATC CGT GGT TTT     1060
Val Asn Val Tyr Tyr Lys Asp Pro Asn Val Leu Pro Ile Arg Gly Phe
```

330 335 340

GGG CTT TTG ATT CCA TCA TGC ACT CCA AAT AAT CCG AAT CAT GTT CTT 1108
Gly Leu Leu Ile Pro Ser Cys Thr Pro Asn Asn Pro Asn His Val Leu
345 350 355 360

GGT ATC GTT TTT GAT AGT GAG CAA AAC AAC CCT GAA AAT GGA AGC AAG 1156
Gly Ile Val Phe Asp Ser Glu Gln Asn Asn Pro Glu Asn Gly Ser Lys
365 370 375

GTC ACT GTC ATG ATG GGA GGG TCT GCT TAT ACA AAA AAT ACT TCT TTG 1204
Val Thr Val Met Met Gly Gly Ser Ala Tyr Thr Lys Asn Thr Ser Leu
380 385 390

ATT CCA ACC AAC CCC GAA GAA GCC GTT AAC AAT GCT CTC AAA GCT TTG 1252
Ile Pro Thr Asn Pro Glu Glu Ala Val Asn Asn Ala Leu Lys Ala Leu
395 400 405

CAG CAT ACT TTA AAA ATA TCC AGT AAG CCA ACA CTC ACG AAT GCA ACA 1300
Gln His Thr Leu Lys Ile Ser Ser Lys Pro Thr Leu Thr Asn Ala Thr
410 415 420

TTA CAA CCA AAT TGC ATC CCT CAA TAT CGT GTT GGG CAT CAA GAT AAT 1348
Leu Gln Pro Asn Cys Ile Pro Gln Tyr Arg Val Gly His Gln Asp Asn
425 430 435 440

CTT AAT TCT TTG AAA TCT TGG ATT GAG AAA AAT ATG GGA GGG CGA ATT 1396
Leu Asn Ser Leu Lys Ser Trp Ile Glu Lys Asn Met Gly Gly Arg Ile
445 450 455

CTT CTA ACT GGA AGT TGG TAT AAT GGT GTT AGT ATT GGG GAT TGT ATT 1444
Leu Leu Thr Gly Ser Trp Tyr Asn Gly Val Ser Ile Gly Asp Cys Ile
460 465 470

ATG AAT GGA CAT TCA ACA GCT CGA AAA CTA GCA TCA TTG ATG AAT TCT 1492
Met Asn Gly His Ser Thr Ala Arg Lys Leu Ala Ser Leu Met Asn Ser
475 480 485

TCT TCT TGAGCGTTTA TAAATGTTGA TATAAAATTA GTATATAGTT CCTTTGATTA 1548
Ser Ser
490

~~TTTTATGAGT TGAAAATGCC ACTTGTGAAA TAATTTTGCA GAAGCCCTTT TATTAGAGAG 1608~~

GTATATGCGA GGACATTCGA CAAACGTTTG AAATTAAAAA TCATATGCCT TTTAGCTTAA 1668

GACATCAAGG TCATGAATAA TAAAATTTT 1697

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 490 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Met Ser Ile Ala Ile Cys Gly Gly Gly Ile Ala Gly Leu Ser Thr Ala
 1               5                  10                  15

Phe Tyr Leu Ala Arg Leu Ile Pro Lys Cys Thr Ile Asp Leu Tyr Glu
            20                  25                  30

Lys Gly Pro Arg Leu Gly Gly Trp Leu Gln Ser Val Lys Ile Pro Cys
        35                  40                  45

Ala Asp Ser Pro Thr Gly Thr Val Leu Phe Glu Gln Gly Pro Arg Thr
    50                  55                  60

Leu Arg Pro Ala Gly Val Ala Gly Leu Ala Asn Leu Asp Leu Ile Ser
65                  70                  75                  80

Lys Leu Gly Ile Glu Asp Lys Leu Leu Arg Ile Ser Ser Asn Ser Pro
                85                  90                  95

Ser Ala Lys Asn Arg Tyr Ile Tyr Tyr Pro Asp Arg Leu Asn Glu Ile
            100                 105                 110

Pro Ser Ser Ile Leu Gly Ser Ile Lys Ser Ile Met Gln Pro Ala Leu
        115                 120                 125

Arg Pro Met Pro Leu Ala Met Met Leu Glu Pro Phe Arg Lys Ser Lys
    130                 135                 140

Arg Asp Ser Thr Asp Glu Ser Val Gly Ser Phe Met Arg Arg Arg Phe
145                 150                 155                 160

Gly Lys Asn Val Thr Asp Arg Val Met Ser Ala Met Ile Asn Gly Ile
                165                 170                 175

Tyr Ala Gly Asp Leu Asn Asp Leu Ser Met His Ser Ser Met Phe Gly
            180                 185                 190

Phe Leu Ala Lys Ile Glu Lys Lys Tyr Gly Asn Ile Thr Leu Gly Leu
        195                 200                 205

Ile Arg Ala Leu Leu Ala Arg Glu Ile Leu Ser Pro Ala Glu Lys Ala
    210                 215                 220

Leu Glu Ser Ser Thr Thr Arg Arg Ala Lys Asn Ser Arg Ala Val Lys
225                 230                 235                 240
```

Gln Tyr Glu Ile Asp Lys Tyr Val Ala Phe Lys Glu Gly Ile Glu Thr
245 250 255

Ile Thr Leu Ser Ile Ala Asp Glu Leu Lys Lys Met Pro Asn Val Lys
260 265 270

Ile His Leu Asn Lys Pro Ala Gln Thr Leu Val Pro His Lys Thr Gln
275 280 285

Ser Leu Val Asp Val Asn Gly Gln Ala Tyr Glu Tyr Val Val Phe Ala
290 295 300

Asn Ser Ser Arg Asn Leu Glu Asn Leu Ile Ser Cys Pro Lys Met Glu
305 310 315 320

Thr Pro Thr Ser Ser Val Tyr Val Val Asn Val Tyr Tyr Lys Asp Pro
325 330 335

Asn Val Leu Pro Ile Arg Gly Phe Gly Leu Leu Ile Pro Ser Cys Thr
340 345 350

Pro Asn Asn Pro Asn His Val Leu Gly Ile Val Phe Asp Ser Glu Gln
355 360 365

Asn Asn Pro Glu Asn Gly Ser Lys Val Thr Val Met Met Gly Gly Ser
370 375 380

Ala Tyr Thr Lys Asn Thr Ser Leu Ile Pro Thr Asn Pro Glu Glu Ala
385 390 395 400

Val Asn Asn Ala Leu Lys Ala Leu Gln His Thr Leu Lys Ile Ser Ser
405 410 415

Lys Pro Thr Leu Thr Asn Ala Thr Leu Gln Pro Asn Cys Ile Pro Gln
420 425 430

Tyr Arg Val Gly His Gln Asp Asn Leu Asn Ser Leu Lys Ser Trp Ile
435 440 445

Glu Lys Asn Met Gly Gly Arg Ile Leu Leu Thr Gly Ser Trp Tyr Asn
450 455 460

Gly Val Ser Ile Gly Asp Cys Ile Met Asn Gly His Ser Thr Ala Arg
465 470 475 480

Lys Leu Ala Ser Leu Met Asn Ser Ser Ser
485 490

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: oligonucleotide used to construct pCGN1761ENX

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AATTATGACG TAACGTAGGA ATTAGCGGCC CGCTCTCGAG T 41

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: oligonucleotide used to construct pCGN1761ENX

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AATTACTCGA GAGCGGCCGC GAATTCCTAC GTTACGTCAT 40

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: primer SON0003 used to construct pSOG10

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CTCGGATCCAGCAGATTCGAAGAAGGTACAG 31

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: primer SON0004 used to construct pSOG10

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

ACGGGATCCAACTTCCTAGCTGAAAAATGGG 31

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: primer SON0031 used to construct pSOG19

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CATGAGGGACTGACCACCCGGGGATC 26

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: primer SON0010 used to construct pSOG19

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AGCGGATAACAATTTCACACAGGA 24

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: primer SON0016 used to construct pSOG19

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GCTACCATGGCCACATAGAACACC 24

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: primer SON0017 used to construct pSOG19

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CGAGAGCTCGCACTTCAACCTTG 23

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: primer SON0039 used to construct pSOG30

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CGACATGGTACGTCCTGTAGAAACCCACA 28

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: primer SON0041 used to construct pSOG30

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ATCGCAAGACCGGCAACAGGATTC 24

We claim:

1. An isolated DNA molecule encoding a protein from a eukaryote having protoporphyrinogen oxidase(protox) activity.

2. The isolated DNA molecule of claim 1 wherein said eukaryote is a higher eukaryote.

3. The isolated DNA molecule of claim 2 wherein said higher eukaryote is a plant.

4. The isolated DNA molecule of claim 3, wherein said plant is a dicotyledon.

5. The isolated DNA molecule of claim 4, wherein said dicotyledon is an *Arabidopsis* species.

6. The isolated DNA molecule of claim 5, wherein said protein comprises the amino acid sequence selected from the group consisting of SEQ ID Nos. 2 and 4.

7. The isolated DNA molecule of claim 3, wherein said plant is a monocotyledon.

8. The isolated DNA molecule of claim 7, wherein said monocotyledon is maize.

9. The isolated DNA molecule of claim 8, wherein said protein comprises the amino acid sequence selected from the group consisting of SEQ ID Nos. 6 and 8.

10. A DNA molecule encoding a modified protoporphyrinogen oxidase(protox) comprising a eukaryotic protox having at least one amino acid modification, wherein said modified protox is tolerant to a herbicide in amounts which inhibit said eukaryotic protox.

11. The DNA molecule of claim 10 wherein said eukaryotic protox is from a plant.

12. The DNA molecule of claim 11 wherein said plant is a dicotyledon.

13. The DNA molecule of claim 12, wherein said dicotyledon is an *Arabidopsis* species.

14. The DNA molecule of claim 13, wherein said eukaryotic protox comprises the amino acid sequence selected from the group consisting of SEQ ID Nos. 2 and 4.

15. The DNA molecule of claim 14, wherein said eukaryotic protox comprises the amino acid sequence set forth in SEQ ID No. 2.

16. The DNA molecule of claim 15, wherein said at least one amino acid modification is a substitution of an amino acid selected from the group consisting of the alanine at position 220, the glycine at position 221 and the tyrosine at position 426 of SEQ ID No. 2.

17. The DNA molecule of claim 16, wherein said alanine at position 220 is replaced with an amino acid selected from the group consisting of valine, threonine, leucine, cysteine and tyrosine.

18. The DNA molecule of claim 16, wherein said glycine at position 221 is replaced with a serine.

19. The DNA molecule of claim 16 wherein said tyrosine at position 426 is replaced with an amino acid selected from the group consisting of cysteine, isoleucine, leucine and threonine.

20. The DNA molecule of claim 11, wherein said plant is a monocotyledon.

21. The DNA molecule of claim 20, wherein said monocotyledon is maize.

22. The DNA molecule of claim 21, wherein said eukaryotic protox comprises the amino acid sequence selected from the group consisting of SEQ ID Nos. 6 and 8.

23. The DNA molecule of claim 22, wherein said eukaryotic protox comprises the amino acid sequence set forth in SEQ ID No. 6.

24. The DNA molecule of claim 23, wherein said at least one amino acid modification is a substitution of an amino acid selected from the group consisting of the alanine at position 166, the glycine at position 167 and the tyrosine at position 372 of SEQ ID No. 6.

25. The DNA molecule of claim 24, wherein said alanine at position 166 is replaced with an amino acid selected from the group consisting of valine, threonine, leucine, cysteine and tyrosine.

26. The DNA molecule of claim 24, wherein said glycine at position 167 is replaced with a serine.

27. The DNA molecule of claim 24 wherein said tyrosine at position 372 is replaced with an amino acid selected from the group consisting of cysteine, isoleucine, leucine and threonine.

28. The DNA molecule according to any one of claims 10 to 27 which is part of a plant genome.

29. A chimeric gene comprising a promoter operably linked to a heterologous DNA molecule encoding a protein from a higher eukaryote having protoporphyrinogen oxidase(protox) activity.

30. The chimeric gene of claim 29 wherein said promoter is active in a plant.

31. The chimeric gene of claim 30 additionally comprising a signal sequence operably linked to said DNA molecule, wherein said signal sequence is capable of targeting the protein encoded by said DNA molecule into the chloroplast.

32. The chimeric gene of claim 30 additionally comprising a signal sequence operably linked to said DNA molecule, wherein said signal sequence is capable of targeting the protein encoded by said DNA molecule into the mitochondria.

33. A chimeric gene comprising a promoter which is active in a plant operably linked to the DNA molecule of claim 10.

34. The chimeric gene of claim 33 additionally comprising a signal sequence operably linked to said DNA molecule, wherein said signal sequence is capable of targeting the protein encoded by said DNA molecule into the chloroplast.

35. The chimeric gene of claim 34 additionally comprising a signal sequence operably linked to said DNA molecule, wherein said signal sequence is capable of targeting the protein encoded by said DNA molecule into the mitochondria.

36. The chimeric gene according to any one of claims 29 to 35 which is part of a plant genome.

37. A recombinant vector comprising the chimeric gene of any one of claims 29 to 35, wherein said vector is capable of being stably transformed into a host cell.

38. A recombinant vector comprising the chimeric gene of claim 33, wherein said vector is capable of being stably transformed into a plant cell.

39. A host cell stably transformed with a vector according to any one of claims 37 or 38, wherein said host cell is capable of expressing said DNA molecule.

40. A host cell of claim 39 selected from the group consisting of a plant cell, a bacterial cell, a yeast cell, and an insect cell.

41. A plant or plant cell including the progeny thereof comprising a DNA molecule of any one of claims 10 to 28 , wherein said DNA molecule is expressed in said plant and confers upon said plant and plant cell, respectivly, tolerance to a herbicide in amounts which inhibit naturally occurring protox activity

42. A plant or plant cell including the progeny thereof comprising a chimeric gene of any one of claims 29 to 36, wherein said chimeric gene confers upon said plant and plant cell, respectively, tolerance to a herbicide in amounts which inhibit naturally occurring protox activity.

43. A plant and its progeny including parts thereof having altered protoporphyrinogen oxidase(protox) activity, wherein said altered protox activity confers upon said plant and its progeny tolerance to a herbicide in amounts which inhibit naturally occurring protox activity.

44. The plant of any one of claims 41 to 43, wherein said plant is a dicotyledon.

45. The plant of claim 44, wherein said plant is selected from the group consisting of soybean, cotton, tobacco, sugar beet, and oilseed rape.

46. The plant of any one of claims 41 to 43, wherein said plant is a monocot.

47. The plant of claim 46, wherein said plant is selected from the group consisting of maize, wheat, sorghum, rye, oats, turf grass and rice.

48. The plant of any one of claims 41 to 43, wherein said altered protox activity is conferred by over-expression of a protox enzyme which naturally occurs in said plant.

49. The plant of any one of claims 41 to 43, wherein said altered protox activity is conferred by expression of a DNA molecule encoding a herbicide tolerant protox enzyme.

50. The plant of claim 49, wherein said herbicide tolerant protox enzyme naturally occurs in a prokaryote.

51. The plant of claim 50 wherein said prokaryote is selected from the group consisting of
E. coli, B. subtilis and *S. typhimurium.*

52. The plant of claim 49 wherein said herbicide tolerant protox enzyme is a modified form of a protein which naturally occurs in a prokaryote.

53. The seed of a plant according to any one of claims 41 to 52.

54. A plant according to any one of claims 41 to 52, which is a hybrid plant.

55. Propagating material of a plant according to any one of claims 41 to 54 treated with a protectant coating.

56. Propagating material according to claim 55, comprising a preparation selected from the group consisting of herbicides, insecticides, fungicides, bactericides, nematicides, molluscicides or mixtures thereof.

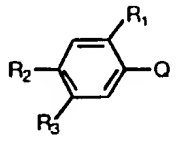
57. Propagating material according to claim 55 or 56 characterized in that it consists of seed.

58. A method for controlling the growth of undesired vegetation which comprises applying to a population of the plant of any one of claims 41 to 54 an effective amount of a protox-inhibiting herbicide.

59. The method of claim 58 wherein said plant is selected from the group consisting of soybean, cotton, tobacco, sugar beet, oilseed rape, maize, wheat, sorghum, rye, oats, turf grasses and rice.

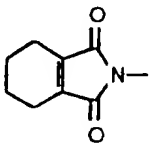
60. The method of claim 59 wherein said protox-inhibiting herbicide is selected from the group consisting of an aryluracil, a diphenylether, an oxidiazole, an imide, a phenyl pyrazole, a pyridine derivative, a 3-substituted-2-aryl-4,5,6,7-tetrahydroindazole, a phenopylate and *O*-phenylpyrrolidino- and piperidinocarbamate analogs of said phenopylate.

61. The method of claim 60 wherein said protox-inhibiting herbicide is an imide having the formula



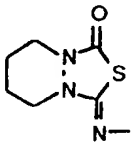
(Formula V)

wherein Q equals



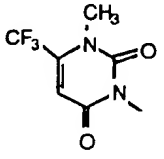
(Formula VI)

OR



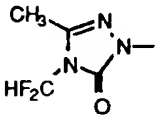
(Formula VII)

OR

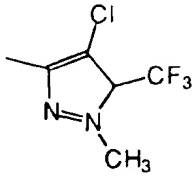


(Formula VIII)

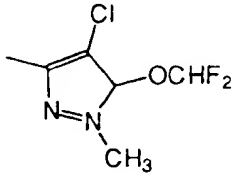
OR



(Formula IX)



OR

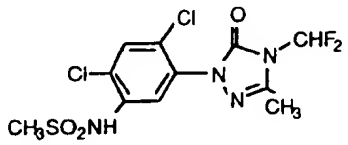


OR

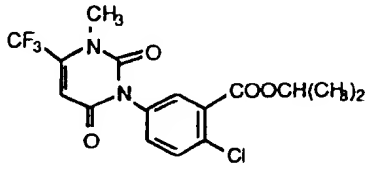
(Formula IXa) (Formula IXb)

and wherein R_1 equals H, Cl or F, R_2 equals Cl and R_3 is an optimally substituted ether, thioether, ester, amino or alkyl group, and wherein R_2 and R_3 together may form a 5 or 6 membered heterocyclic ring.

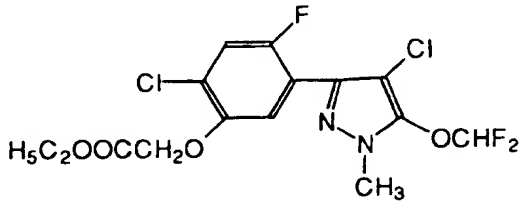
62. The method of claim 61 wherein said imide is selected from the group consisting of



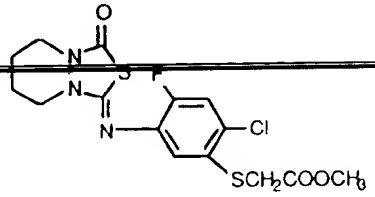
(Formula X);



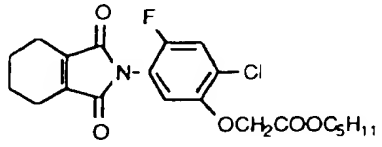
(Formula XI);



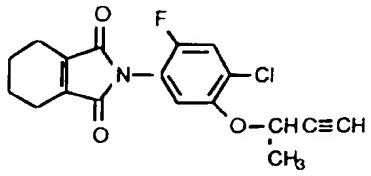
(Formula XII);



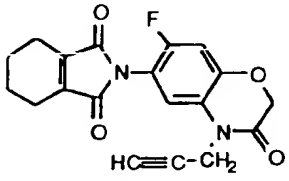
(Formula XIII);



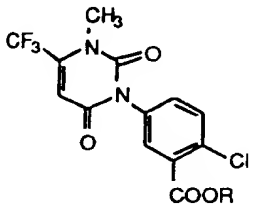
(Formula XIV);



(Formula XV);



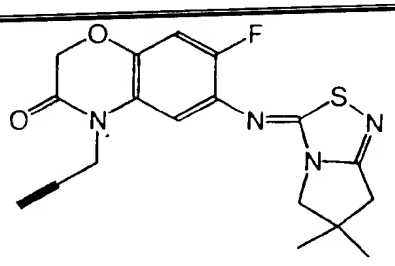
(Formula XVI); and



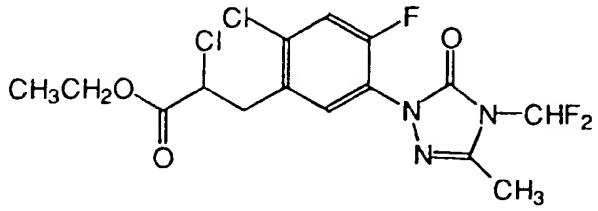
(Formula XVII)

wherein R signifies (C_{2-6}-alkenyloxy)carbonyl-C_{1-4}-alkyl.

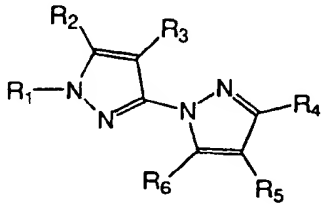
63. The method of claim 58 wherein said protox-inhibiting herbicide has the formula selected from the group consisitng of



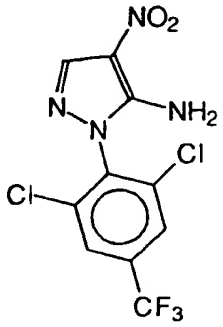
(Formula XVIII),



(Formula XIX),



(Formula XX), and



(Formula XXI).

64. A method for assaying a chemical for the ability to inhibit the activity of a protox enzyme from a plant comprising

(a) combining said protox enzyme and protoporphyrinogen IX in a first reaction mixture under conditions in which said protox enzyme is capable of catalyzing the conversion of said protoporphyrinogen IX to protoporphyrin IX;

(b) combining said chemical, said protox enzyme and protoporphyrinogen IX in a second reaction mixture under the same conditions as in said first reaction mixture;

(c) exciting said first and said second reaction mixtures at about 395 to about 410 nM;

(d) comparing the flourescence of said first and said second reaction mixtures at about 622 to about 635 nM;

wherein said chemical is capable of inhibiting the activity of said protox enzyme if the flourescence of said second reaction mixture is significantly less than the flourescence of said first reaction mixture.

65. A method of identifying a modified protox enzyme resistant to a protox inhibitor present in a population of cells comprising the steps of

(a) culturing said population in the presence of said protox inhibitor in amounts which inhibit the unmodified form of said protox enzyme;

(b) selecting those cells from step (a) whose growth is not inhibited; and

(c) isolating and identifying the protox enzyme present in the cells selected from step (b).

66. A method of selecting plants, plant tissue or plant cells transformed with a transgene of interest from non-transformed plants, comprising the steps of:

(a) transforming a plant, plant tissue or plant cell with a transgene of interest capable of being expressed by the plant, and a gene encoding an altered protox resistant to a protox inhibitor;

(b) transferring the thus-transformed plants or plant cells to a medium comprising the protox inhibitor; and

(c) selecting the plants or plant cells which survive in the medium.

67. A probe capable of specifically hybridizing to a eukaryotic protoporphyrinogen oxidase gene or mRNA, wherein said probe comprises a contiguous portion of the

coding sequence for a protoporphyrinogen oxidase from a eukaryote at least 10 nucleotides in length.

68. The probe of claim 67 wherein said coding sequence is selected from the group consisting of SEQ ID Nos. 1, 3, 5, 7 and 9.

69. A method of producing a host cell comprising an isolated DNA molecule encoding a protein from a eukaryote having protoporphyrinogen oxidase(protox) activity comprising transforming the said host cell with a recombinant vector molecule according to claim 37 or 38.

70. A method of producing a plant cell comprising an isolated DNA molecule encoding a protein from a eukaryote having protoporphyrinogen oxidase(protox) activity comprising transforming the said plant cell with a recombinant vector molecule according to claim 37 or 38.

71. A method of producing transgenic progeny of a transgenic parent plant comprising an isolated DNA molecule encoding a protein from a eukaryote having protoporphyrinogen oxidase(protox) activity comprising transforming the said parent plant with a recombinant vector molecule according to claim 37 or 38 and transferring the herbicide tolerant trait to the progeny of the said transgenic parent plant involving known plant breeding techniques.

72. A method of producing a DNA molecule encoding a protein from a eukaryote having protoporphyrinogen oxidase(protox) activity comprising

(a) establishing a cDNA library from a suitable eukaryotic source;

(b) identifying cDNA clones encoding a protox enzyme based on their ability to supply protox enzymatic activity to a mutant host organism deficient in this activity.

73. A method of producing a DNA molecule encoding a protein from a eukaryote having protoporphyrinogen oxidase(protox) activity comprising

(a) establishing a genomic or a cDNA library from a suitable eukaryotic source;
(b) probing the said library with a probe molecule according to claim 67.

74. Use of a DNA molecule according to any one of claims 28 or 36 to confer tolerance to a herbicide in amounts which inhibit naturally occurring protox activity from a parent plant to its progeny comprising first stably transforming the parent plant with a DNA molecule according to any one of claims 10 to 27 by stably incorporating the said DNA molecule into the plant genome of the said parent plant and second transferring the herbicide tolerant trait to the progeny of the said transgenic parent plant involving known plant breeding techniques.

75. Use of a DNA molecule according to any one of claims 28 or 36 to prepare a medical tool for the treatment of deficiencies in protoporphyrinogen oxidase(protox) activity in animals, particularly humans.

76. Use of a DNA molecule according to any one of claims 28 or 36 to prepare a medical tool for diagnosing deficiencies in protoporphyrinogen oxidase(protox) activity in animals, particularly humans.

77. A pharmaceutical composition comprising together with a pharmaceutically acceptable carrier a protein obtainable from a eukaryote having protoporphyrinogen oxidase(protox) activity to be used in a method for treatment the animal or human body or for diagnostic purposes.

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/IB 95/00452

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/53 C12N15/82 C12N5/10 A01H5/00 C12Q1/26
A01H1/04 C12Q1/68 A61K38/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H C12Q A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PLANT PHYSIOLOGY, vol. 97, 1991 pages 280-287, SHERMAN, T.D., ET AL. 'Physiological basis for differential sensitivities of plant species to protoporphyrinogen oxidase-inhibiting herbicides' see page 282, left column ---	64
X	FEBS LETTERS, vol. 245, no. 1,2, March 1989 AMSTERDAM NL, pages 35-38, MATRINGE, M., ET AL. 'PROTOPORPHYRINOGEN OXIDASE INHIBITION BY THREE PEROXIDIZING HERBICIDES: OXADIAZON, LS 82-556 AND M&B 39279' see the whole document --- -/--	64

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search
8 September 1995

Date of mailing of the international search report
13. 10. 95

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	INTERNATIONAL WORKSHOP ON CHLOROPLAST METABOLISM AND ITS INHIBITION BY HERBICIDES, OMIYA, JAPAN, AUGUST 26-28, 1992. Z NATFORSCH SECT C BIOSCI 48 (3-4). 1993. 339-344., OSHIO H ET AL 'ISOLATION AND CHARACTERIZATION OF A CHLAMYDOMONAS-REINHARDTII MUTANT RESISTANT TO PHOTOBLEACHING HERBICIDES.'	65
A	see the whole document	10
X	EMBL SEQUENCE DATABASE ACC. NO M22063 REL.19, 22-APR-1989, see sequence 3683-3695 identical to 1587-1575 in reverse strand of seq. i.d. 1	67
P,X	EMBL SEQUENCE DATABASE ACC. NO. T43573, REL. NO. 42, 3-FEB-1995 see sequence 7-483	67
P,X	PESTIC. BIOCHEM. PHYSIOL. (1994), 50(2), 107-14, PORNPROM, TOSAPON 'Characterization of oxyfluorfen tolerance in selected soybean cell' see the whole document	65
P,X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 14, 7 April 1995 MD US, pages 8076-8080, NISHIMURA, K., TE AL. 'CLONING OF A HUMAN CDNA FOR PROTOPORPHYRINOGEN OXIDASE BY COMPLEMENTATION IN VIVIO OF A HEMG MUTANT OF ESCHERICHIA COLI' see the whole document	72
A	JOURNAL OF BIOLOGICAL CHEMISTRY 269 (2). 1994. 813-815. , DAILEY T A ET AL 'Expression of a cloned protoporphyrinogen oxidase.' see the whole document	1
A	J. BACTERIOLOGY, vol. 174, 1992 pages 8081-8093, HANSSON, M., ET AL. 'CLONING AND CHARACTERIZATION OF THE BACILLUS SUBTILIS HEMEHY GENE CLUSTER, WHICH ENCODES PROTOHEME IX BIOSYNTHETIC ENZYMES' see page 8090 - page 8092	1

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INTERNATIONAL SEARCH REPORT

Intern ıal Application No
PCT/IB 95/00452

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BIOCHEMICAL JOURNAL, vol. 244, 1987 pages 219-224, JACOBS, J.M., ET AL. 'OXIDATION OF PROTOPORPHYRINOGEN TO PROTOPHORPHYRIN, A STEP IN CHLOROPHYLL AND HAEM BIOSYNTHESIS' see the whole document ---	1
A	CANADIAN JOURNAL OF MICROBIOLOGY 39 (12). 1993. 1155-1161., SASARMAN A ' Nucleotide sequence of the hemG gene involved in the protoporphyrinogen oxidase activity of Escherichia coli K12.' see the whole document ---	1
A	ACS SYMP. SER. (1994), 559(PORPHYRIC PESTICIDES), 191-204, DUKE, STEPHEN O. 'Protoporphyrinogen oxidase as the optimal herbicide site in the porphyrin pathway' see the whole document ---	1
A	EP,A,0 478 502 (CIBA GEIGY AG) 1 April 1992 see the whole document ---	1-77
A	WEED RESEARCH, vol. 39, no. 2, 1994 pages 102-108, PORNPROM, T., ET AL. 'SELECTION FOR HERBICIDE TOLERANCE IN SOYBEAN USING CELL SUSPENSION CULTURE' see the whole document ---	65
A	PLANT MOLECULAR BIOLOGY, vol. 23, 1993 pages 35-43, MADSEN, O., ET AL. 'A SOYBEAN COPROPORPHYRINOGEN OXIDASE GENE IS HIGHLY EXPRESSED IN ROOT NODULES' see the whole document -----	1

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern al Application No
PCT/IB 95/00452

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0478502	01-04-92	US-A-	5290926	01-03-94
		AU-A-	8386791	19-03-92
		CA-A-	2051240	15-03-92
		JP-A-	5068544	23-03-93